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WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 5 :
C12N 15/53, 15/82, 9/06
C12N 9/02, 5/10, A01H 5/00
A01H 1/04

A1

(11) International Publication Number: WO 92/00377

(43) International Publication Date: 9 January 1992 (09.01.92)

(21) International Application Number: PCT/US91/04514

(22) International Filing Date: 24 June 1991 (24.06.91)

(30) Priority data:

543,236	25 June 1990 (25.06.90)	US
717,370	24 June 1991 (24.06.91)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), SU.

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GLYPHOSATE TOLERANT PLANTS

(57) Abstract

Genes encoding a glyphosate oxidoreductase enzyme are disclosed. The genes are useful in producing transformed bacteria and plants which degrade glyphosate herbicide as well as crop plants which are tolerant to glyphosate herbicide.

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GLYPHOSATE TOLERANT PLANTS

This is a continuation-in-part of our co-pending application having serial number 07/543,236 which was filed on June 25, 1990.

BACKGROUND OF THE INVENTION

Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. It is now possible to produce plants which have unique characteristics of agronomic importance. Certainly, one such advantageous trait is more cost effective, environmentally compatible weed control via herbicide tolerance. Herbicide-tolerant plants may reduce the need for tillage to control weeds thereby effectively reducing soil erosion.

One herbicide which is the subject of much investigation in this regard is N-phosphonomethyl-glycine commonly referred to as glyphosate. Glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids and vitamins. Specifically, glyphosate inhibits the conversion of phosphoenolpyruvic acid and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSP synthase or EPSPS).

It has been shown that glyphosate tolerant plants can be produced by inserting into the genome of the plant the capacity to produce a higher level of EPSP synthase which enzyme is preferably glyphosate tolerant (Shah et al., 1986). The introduction into plants of glyphosate degradation gene(s) could provide a means of conferring glyphosate tolerance to plants and/or to augment the tolerance of transgenic plants

already expressing a glyphosate tolerant EPSP synthase depending upon the physiological effects of the degradation products.

Glyphosate metabolism (degradation) has been examined in a wide variety of plants and little degradation has been reported in most of those studies. In those instances where degradation has been reported, the initial breakdown product is usually aminomethylphosphonate (AMPA) (Coupland, 1985; Marshall et al., 1987). In these instances, it is not clear if glyphosate is metabolized by the plant or the contaminating microbes on the leaf surface to which glyphosate was applied. AMPA has been reported to be much less phytotoxic than glyphosate for most plant species (Franz, 1985) but not for all plant species (Maier, 1983; Tanaka et al., 1988). Glyphosate degradation in soils is much more extensive and rapid (Torstensson, 1985). The principal breakdown product identified is AMPA (Rueppel et al., 1977; Nomura and Hilton, 1977); a phosphonate that can be metabolized by a wide variety of microorganisms (Zeleznick et al., 1963; Mastalerz et al., 1965; Cook et al., 1978; Daughton et al., 1979a; 1979b; 1979c; Wackett et al., 1987a). A number of pure cultures of bacteria have been identified that degrade glyphosate by one of the two known routes (Moore et al., 1983; Talbot et al., 1984; Shinabarger and Braymer, 1986; Balthazor and Hallas, 1986; Kishore and Jacob, 1987; Wackett et al., 1987a; Pipke et al., 1987a; Pipke et al., 1987b; Hallas et al., 1988; Jacob et al., 1985 and 1988; Pipke and Amrhein, 1988; Quinn et al., 1988 and 1989; Lerbs et al., 1990; Schowanek and Verstraete, 1990; Weidhase et al., 1990; Liu et al., 1991). A route involving a "C-P lyase" that degrades glyphosate to sarcosine and inorganic orthophosphate (Pi) has been reported for a *Pseudomonas* sp. (Shinabarger and Braymer, 1986; Kishore and Jacob, 1987) and an *Arthrobacter* sp. (Pipk et al., 1987b). Pure cultures capabl of degrading

glyphosate to AMPA have been reported for a *Flavobacterium* sp. (Balthazor and Hallas, 1986), for a *Pseudomonas* sp. (Jacob et al., 1988) and for *Arthrobacter atrocyaneus* (Pipke and Amrhein, 1988). In addition, a large number of isolates that convert glyphosate to AMPA have been identified from industrial activated sludges that treat glyphosate wastes (Hallas et al., 1988). However, the number and nature of bacterial genes responsible for these degradations have not been heretofore determined nor have the gene(s) been isolated.

Hence, in one aspect, an object of the present invention is to provide novel genes which encode a glyphosate metabolizing enzyme which converts glyphosate to aminomethylphosphonate and glyoxylate.

Another object is to enhance the activity of the glyphosate metabolizing enzyme against glyphosate by replacement of specific amino acid residues.

Another object of the present invention is to provide genetically modified plants which express a gene which encodes a glyphosate metabolizing enzyme and which exhibit enhanced tolerance to glyphosate herbicide.

Another object is to demonstrate that a glyphosate metabolizing enzyme can be targeted to plastids using chloroplast transit peptides and the plastid targeted enzyme confers high level glyphosate tolerance.

A further object is to provide a method for selecting transformed plant tissue using the glyphosate metabolizing enzyme as the selectable marker in the presence of inhibitory concentrations of glyphosate.

These and other objects, aspects and features of the present invention will become evident to those skilled in the art from the following description and working examples.

SUMMARY OF THE INVENTION

The present invention provides structural DNA constructs which encode a glyphosate oxido-reductase enzyme and which are useful in producing glyphosate degradation capability in heterologous microorganisms (e.g. bacteria and plants) and in producing glyphosate tolerant plants.

In accomplishing the foregoing, there is provided, in accordance with one aspect of the present invention, a method of producing genetically transformed plants which are tolerant toward glyphosate herbicide, comprising the steps of:

(a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising
   (i) a promoter which functions in plant cells to cause the production of an RNA sequence,
   (ii) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxidoreductase enzyme,
   (iii) a 3' non-translated DNA sequence which functions in plant cells to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence;

   where the promoter is heterologous with respect to the coding sequence and adapted to cause sufficient expression of said enzyme in plant tissue, including meristematic tissue, to enhance the glyphosate resistance of a plant cell transformed with said gene;

(b) obtaining a transformed plant cell; and

(c) regenerating from the transformed plant cell a genetically transformed plant which has increased tolerance to glyphosate herbicide.

In accordance with another aspect of the present invention, there is provided a recombinant, double-stranded DNA molecule comprising in sequence:

(a) a promoter which functions in plants to cause the production of an RNA sequence;
(b) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxidoreductase enzyme; and
(c) a 3' non-translated region which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence.

There has also been provided, in accordance with another aspect of the present invention, bacterial and transformed plant cells that contain, respectively, DNA comprised of the above-mentioned elements (a), (b) and (c).

In accordance with yet another aspect of the present invention, differentiated plants have been provided that comprise transformed plant cells, as described above, which exhibit tolerance toward glyphosate herbicide.

In accordance with still another aspect of the present invention, there has been provided a method for selectively controlling weeds in a field containing a crop having planted crop seeds or plants comprising the steps of:

(a) planting said crop seeds or plants which are glyphosate tolerant as a result of a recombinant double-stranded DNA molecule being inserted into said crop seed or plant, said DNA molecule having
    (i) a promoter sequence which functions in plants to cause the production of an RNA sequence,
    (ii) a structural DNA sequence which causes the production of RNA which encodes a glyphosate oxidoreductase enzyme,

(iii) a 3' non-translated region which encodes a polyadenylation signal which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence,

where the promoter is heterologous with respect to the coding sequence and adapted to cause sufficient expression of said enzyme in plant tissue, including meristematic tissue, to enhance the glyphosate tolerance of a plant cell transformed with said gene; and

(b) applying to said crop and weeds in said field a sufficient amount of glyphosate herbicide to control said weeds without significantly affecting said crop.

In a particularly preferred embodiment the double-stranded DNA molecule comprising a gene for plant expression comprises a structural DNA sequence encoding a fusion polypeptide containing an amino- terminal chloroplast transit peptide which is capable of causing importation of the carboxy-terminal glyphosate oxidoreductase enzyme into the chloroplast of the plant cell expressing said gene.

A further embodiment of the present invention is the use of the glyphosate oxidoreductase gene as a selectable marker to select and identify transformed plant tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA sequence for the full-length promoter of figwort mosaic virus (FMV).

Figure 2 shows the structural DNA sequence for a glyphosate oxidoreductase gene from bacterial isolate LBAA.

Figure 3 shows a comparison of the manipulated structural glyphosate oxidoreductase gene versus a modified

glyphosate oxidoreductase gene adapted for enhanced expression in plants. The manipulated glyphosate oxidoreductase gene is displayed as the upper DNA sequence. Only the changes made in the modified gene are indicated in the lower strand of sequences.

Figure 4 shows a comparison of the manipulated structural glyphosate oxidoreductase gene versus a synthetic glyphosate oxidoreductase gene adapted for enhanced expression in plants. The manipulated glyphosate oxidoreductase gene is displayed as the upper DNA sequence.

Figure 5 shows the structure of pMON17032, a pMON886 vector containing the modified glyphosate oxidoreductase gene inserted as an En-CaMV35S-modified glyphosate oxidoreductase-NOS 3' cassette into the *Not*I site of the vector. The pMON886 vector is described in the text.

Figure 6 shows the nucleotide sequence of the CTP1 chloroplast transit peptide derived from the *A. thaliana* SSU1A gene.

Figure 7 shows the genetic/structural map of plasmid pMON17066, a pMON979-type vector containing a gene encoding a CTP/synthetic glyphosate oxidoreductase fusion polypeptide. Related pMON979-type derivatives are pMON17065 and pMON17073.

Figure 8 shows the genetic/structural map of plasmid pMON17138, an example of a pMON981-type vector containing a gene encoding a CTP/synthetic glyphosate oxidoreductase fusion polypeptide. In this example the CTP1-synthetic glyphosate oxidoreductase gene has been cloned into pMON979 as a *Xba*I-*Bam*HI fragment.

Figure 9 shows the nucleotide sequence of the CTP2 chloroplast transit peptide derived from the *A. thaliana* EPSPS gene.

Figure 10 shows the structural map of plasmid pMON17159.

Figure 11 shows the structural map of plasmid pMON17226.

Figure 12 shows the structural map of plasmid pMON17164.

STATEMENT OF THE INVENTION

The expression of a plant gene which exists in double-stranded DNA form involves synthesis of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' signal region which facilitates addition of polyadenylate nucleotides to the 3' end of the RNA.

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription into mRNA using one of the DNA strands as a template to make a corresponding complementary strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters and the figwort mosaic virus (FMV) 35S promoter, the light-inducible promoter from the small subunit of ribulose bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create

various types of DNA constructs which have been expressed in plants; see, e.g., PCT publication WO 84/02913 (Rogers et al., Monsanto).

Promoters which are known or are found to cause transcription of DNA in plant cells can be used in the present invention. Such promoters may be obtained from a variety of sources such as plants and plant DNA viruses and include, but are not limited to, the CaMV35S and FMV35S promoters and promoters isolated from plant genes such as ssRUBISCO genes or the chlorophyll a/b binding proteins. As described below, it is preferred that the particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of glyphosate oxidoreductase to render the plant substantially tolerant to glyphosate herbicides. The amount of glyphosate oxidoreductase needed to induce the desired tolerance may vary with the plant species.

It is preferred that the promoters utilized have relatively high expression in all meristematic tissues in addition to other tissues inasmuch as it is now known that glyphosate is translocated and accumulated in this type of plant tissue. Alternatively, a combination of chimeric genes can be used to cumulatively result in the necessary overall expression level of glyphosate oxidoreductase enzyme to result in the glyphosate tolerant phenotype.

The mRNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs, as presented in the following examples, wherein the non-translated region is

derived from both the 5' non-translated sequence that accompanies the promoter sequence and part of the 5' non-translated region of the virus coat protein gene. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence as discussed above.

A preferred promoter for use in the present invention is the full-length transcript (35S) promoter from the figwort mosaic virus (FMV) which functions as a strong and uniform promoter for chimeric genes inserted into plants, particularly dicotyledons. In general, the resulting transgenic plants express the protein encoded by the inserted gene at a higher and more uniform level throughout the tissues and cells than the same gene driven by an enhanced CaMV35S promoter. Referring to Figure 1, the DNA sequence of the promoter is located between nucleotides 6368 and 6930 (SEQ ID NO:1) of the FMV genome. A 5' non- translated leader sequence is preferably coupled with the promoter and an exemplary leader sequence (SEQ ID NO:2) is shown in Figure 1. The leader sequence can be from the FMV genome itself or can be from a source other than FMV.

The 3' non-translated region of the chimeric plant gene contains a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene. An example of a preferred 3' region is that from the ssRUBISCO gene from pea (E9), described in greater detail in the examples below.

The DNA constructs of the present invention also contain a structural coding sequence in double-stranded DNA form, which encodes a glyphosate oxidoreductase enzyme which converts glyphosate to aminomethylphosphonate and glyoxylate.

Summary of the Glyphosate Oxidoreductase Reaction

The enzyme glyphosate oxidoreductase catalyzes the cleavage of the C-N bond of glyphosate yielding aminomethyl phosphonate (AMPA) and glyoxylate as the reaction products. Under aerobic conditions, oxygen is utilized as a cosubstrate for the reaction. Other electron carriers such as phenazine methosulfate and ubiquinone stimulate the reaction under aerobic conditions. In the absence of oxygen, these compounds act as electron acceptors.

The enzymatic reaction can be assayed by oxygen uptake using an oxygen electrode. The glyphosate oxidoreductase from LBAA does not produce hydrogen peroxide as a product of oxygen reduction. This enzyme has a stoichiometry of two moles of glyphosate oxidized per mole of oxygen consumed and produces two moles each of AMPA and glyoxylate as reaction products.

An alternate method for the assay of glyphosate oxidoreductase involves reaction of the sample with 2,4-dinitrophenylhydrazine and determination of the amount of the glyoxylate-2,4-dinitrophenylhydrazone by HPLC analysis as described in detail in a later section.

A third method for the assay of glyphosate oxidoreductase consists of using [3-^{14}C]-glyphosate as a substrate; the radioactive AMPA produced by the enzyme is separated from the substrate by HPLC on anion exchange column as described later. The radioactivity associated witn

AMPA is a measure of the extent of the glyphosate oxidoreductase reaction.

Glyphosate oxidoreductase from LBAA is a flavoprotein using FAD as a cofactor. One of the mechanisms we have proposed for the reaction catalyzed by this enzyme involves the reduction of the FAD at the active site of the enzyme by glyphosate. This leads to the formation of reduced FAD and a Schiff base of aminomethylphosphonate with glyoxylate. The Schiff base is hydrated by water and hydrolyzed to its components, AMPA and glyoxylate. The reduced flavin is reoxidized by molecular oxygen. We suggest that during the process of reoxidation of reduced FAD, an oxygenated flavin is produced as an intermediate. This flavin intermediate may catalyze the oxygenation of glyphosate yielding AMPA and glyoxylate. This hypothesis is in accordance with the observed stoichiometry and our inability to detect hydrogen peroxide in the reaction mixture.

In addition to glyphosate, glyphosate oxidoreductase from LBAA oxidizes iminodiacetic acid (IDA) to glycine and glyoxylate. The rate of the reaction with IDA is significantly faster than with glyphosate.

Isolation of Efficient Glyphosate-to-AMPA Degrading Bacterium

Bacteria capable of degrading glyphosate are known. (Hallas et al., 1988; Malik et al., 1989). A number of these bacteria were screened for the rapid degradation of glyphosate in the following manner: twenty three bacterial isolates were transferred from TSA (Trypticase Soya Agar; BBL) plates into medium A consisting of Dworkin-Foster salts medium containing glucose, gluconate and citrate (each at 0.1%) as carbon source and containing glyphosate at 0.1 mM as the phosphorous source.

Dworkin-Foster minimal medium was made up by combining in 1 liter (with autoclaved H_2O) 1 ml each of A, B and C and 10 ml of D, thiamine HCl (5 mg), C-sources to final concentrations of 0.1% each and P-source (glyphosate or other phosphonates or Pi) to the required concentration:

A. D-F Salts (1000X stock; per 100 ml; autoclaved):

H_3BO_3	1 mg
$MnSO_4.7H_2O$	1 mg
$ZnSO_4.7H_2O$	12.5 mg
$CuSO_4.5H_2O$	8 mg
$NaMoO_3.3H_2O$	1.7 mg

B. $FeSO_4.7H_20$ (1000X stock; per 100 ml; autoclaved)
0.1 g

C. $MgSO_4.7H_2O$ (1000X stock; per 100 ml; autoclaved)
20 g

D. $(NH_4)_2SO_4$ (100X stock; per 100 ml; autoclaved)
20 g

Yeast Extract (YE; Difco) was added to a final concentration of 0.01 or 0.001%.

Each 1 ml of culture medium also contained approximately 200,000 cpm [3-^{14}C]glyphosate (Amersham; CFA.745). The cultures were incubated with shaking at 30°C. Isolate LBAA showed significant growth at day one, while other test cultures showed little growth before day three. Determination of radioactivity (by scintillation counting) in the culture, cell pellet and culture supernatant (at day 4) revealed that total ^{14}C radioactivity had decreased and that remaining was partitioned ~1:1 in the supernatant and pellet, indicating that significant uptake and metabolism of glyphosate had taken place.

TABLE I - Glyphosate Metabolism by LBAA Culture

Sample	^{14}C cpm
control	18,631
LBAA culture	11,327
LBAA supernatant	6,007
LBAA cells	4,932

At day five, 75 µl of the culture supernatant of all test cultures was analyzed by HPLC as follows: a SYNCHROPAK™ AX100 anion exchange column (P.J. Cobert) was used and the mobile phase consisted of 65 mM KH_2PO_4 (pH5.5 with NaOH; depending on the needs of the experiment the concentration of the phosphate buffer was varied from 50 to 75 mM in order to alter the retention times of the material), run isocratically and the eluted material monitored continuously using a radioactive detector. This analysis revealed, in one isolate in particular (LBAA), that the glyphosate peak (Retention Time [RT] = 7.0 minutes in this analysis) was completely absent and a new peak of radioactivity had appeared, with the same RT as methylamine or N-acetylmethylamine (RT = 3.5 minutes). The collection of bacteria, of which strain LBAA formed a part, had been characterized as degrading glyphosate to AMPA (Hallas et al., 1988); the detection of methylamine or N-Acetylmethylamine suggested that the AMPA or N-AcetylAMPA was being metabolized by the LBAA "C-P lyase" activity to release the phosphate required for growth in this experiment. Strain LBAA was examined in greater detail.

Conversion of Glyphosate to AMPA in Microbial Isolates

For clarity and brevity of disclosure, the following description of the isolation of genes encoding glyphosate

oxidoreductase enzymes is directed to the isolation of such a gene from a bacterial isolate (LBAA). Those skilled in the art will recognize that the same or a similar strategy can be utilized to isolate such genes from other microbial isolates.

The glyphosate degradation pathway was characterized in resting cells of glyphosate-grown strain LBAA as follows: the cells from a 100 ml culture of LBAA, grown in DF medium with glucose, gluconate and citrate as carbon sources and with thiamine and Yeast Extract (0.01%) to supply trace requirements (= medium DF3S) and with glyphosate at 0.2 mM as a phosphorous source, were harvested at Klett = 200, washed twice with 20 ml of DF3S medium and the equivalent of 20 ml cells resuspended in 100 ul of the same medium containing [3-^{14}C]glyphosate (2.5 ul of 52 mCi/mmol). The cell mix was incubated at 30°C with shaking and samples (20 ul) were withdrawn at intervals. The samples were centrifuged and both the supernatant and cell pellets were analyzed by HPLC (the cell pellets were resuspended in 100 ul of acid-DF3S [= DF3S, 0.65N HCl], boiled for 5 minutes, centrifuged briefly and this supernatant was analyzed; an acidified glyphosate control was also examined). In two hours the amount of radioactivity in the glyphosate peak (RT = 7.8 minutes) in the supernatant had decreased to ~33% of the starting level; about 3% of the glyphosate was found within the cell. Material co-eluting with the methylamine standard accounted for ~5% of the starting counts in the supernatant and for ~1.5% in the cell pellet. A new peak, accounting for ~1.5% of the starting radioactivity with a RT of 7.7 minutes (glyphosate RT = 8.9 minutes upon acidification in this experiment) was identified in the cell contents. The large decrease in overall radioactivity also suggested that the glyphosate was extensively m tabolized in this experiment. The pathway was elucidated further in a subsequent experiment wher the metabolism f [^{14}C]AMPA

was compared to that of [3-^{14}C]glyphosate (as above) in resting cells harvested at Klett 165 and resuspended at the equivalent to 15 ml cells per 100 ul DF3S medium. The samples were analyzed by HPLC and consisted of whole cultures acidified and treated as described above. Within the first two hours of the glyphosate experiment, 25% of the radioactivity was found in the methylamine/N-acetylmethalamine peak (RT = 4.8 minutes), 12.5% as AMPA (RT = 6.4 minutes), 30% as the peak alluded to above (RT = 9.4 minutes) and 30% as glyphosate (RT = 11.8 minutes). In the AMPA experiment 15% of the radioactivity was found as N–acetylmethylamine/methylamine, 59% as AMPA and 18% in the peak with RT = 9.4 minutes. The modified form of AMPA was identified as N–acetylAMPA. A similar acetylation step has been inferred from the products identified in *E. coli* growing in aminomethylphosphonates as sole sources of P (Avila et al., 1987). These data indicated that the glyphosate degradation pathway in LBAA is glyphosate → AMPA (→ methylamine) → N–acetylAMPA → N–acetylmethylamine.

Cloning of the Glyphosate Oxidoreductase Gene(s) in *E. coli*

Having established the glyphosate-to-AMPA conversion in strain LBAA, a direct approach for the cloning of the gene(s) involved in this conversion into *E. coli* was investigated. Cloning and genetic techniques, unless otherwise indicated, were generally those described (Maniatis et al., 1982). The cloning strategy was as follows: introduction of a cosmid bank of strain LBAA into *E. coli* and selection for the glyphosate-to-AMPA gene(s) by requiring growth on glyphosate as a phosphorous (P) source. This selection relied on the use of AMPA generated by the glyphosate metabolizing enzyme as a P source, following the release of the Pi from the AMPA by the *E. coli* "C-P lyase." Most *E. coli* strains are incapable of

utilizing phosphonates as P sources upon initial challenge, however these strains usually adapt rapidly, independently of RecA, to utilize phosphonates (become Mpu+) (Wackett et al., 1987b). *E. coli* Mpu+ was isolated from *E. coli* SR200 (Leu-, Pro-, *recA*, *hsdR*, *supE*, Sm^r, *tonA*,) as follows: an aliquot of a fresh L-broth culture of *E. coli* SR200 was plated on MOPS (Neidhardt et al., 1974) complete agar (i.e., contains L-leucine and L-proline at 25 ug/ml and vitamin B1 [thiamine] at 10 ug/ml; agar = DIFCO "Purified") containing aminomethylphosphonate (AMPA; 0.2 mM; Sigma) as P source.

MOPS medium is:

10 ml	10X MOPS SALTS
2 ml	0.5 mg/ml Thiamine HCl
1 ml	20% glucose

10 X MOPS Salts are:
for 100 ml

40 ml	1M MOPS pH7.4
4 ml	1M Tricine pH7.4
1 ml	0.01 M $FeSO_4.7H_2O$
5 ml	1.9 M NH_4Cl
1 ml	0.276 M K_2SO_4
1 ml	0.5 mM $CaCl_2$
1 ml	0.528 M $MgCl_2$
10 ml	5 M NaCl
1 ml	0.5% L-Methionine
1 ml	Micronutrients

Micronutrients are:

3×10^{-9} M $(NH_4)_6Mn_7O_{24}$

4×10^{-7} M H_3BO_4

3×10^{-8} M $CoCl_2$

1.6×10^{-8} M $CuSO_4$

8×10^{-8} M $MnCl_2$

1×10^{-8} M $ZnSO_4$

Six individual colonies were picked from this plate after three days incubation at 37°C and streaked on MOPS complete agar containing either AMPA or methylphosphonate (Alfa) as P source. One colony, designated *E. coli* SR200 Mpu+, was chosen from those that grew equally and uniformly on both phosphonate media.

Chromosomal DNA was prepared from strain LBAA as follows: The cell pellet from a 100 ml L-Broth (Miller, 1972) late log phase culture of LBAA was resuspended in 10 ml of Solution I (Birnboim and Doly, 1979). SDS was added to a final concentration of 1% and the suspension was subjected to three freeze-thaw cycles, each consisting of immersion in dry ice for 15 minutes and in water at 70°C for 10 minutes. The lysate was then extracted four times with equal volumes of phenol:chloroform (1:1; phenol saturated with TE) (TE = 10mM Tris pH8.0; 1.0mM EDTA) and the phases separated by centrifugation (15000g; 10 minutes). The ethanol-precipitable material was pelleted from the supernatant by brief centrifugation (8000g; 5 minutes) following addition of two volumes of ethanol. The pellet was resuspended in 5 ml TE and dialyzed for 16 hours at 4°C against 2 liters TE. This preparation yielded a 6 ml DNA solution of 150 μg/ml.

Partially-restricted DNA was prepared as follows: Thre 100 μg aliquot samples of LBAA DNA were treated for 1

hour at 37 C with restriction endonuclease *Hin*dIII at rates of 4, 2 and 1 enzyme unit/µg DNA, respectively. The DNA samples were pooled, made 0.25 mM with EDTA and extracted with equal volume of phenol:chloroform. Following the addition of NaAcetate and ethanol, the DNA was precipitated with two volumes of ethanol and pelleted by centrifugation (12000 g; 10 minutes). The dried DNA pellet was resuspended in 500 µl TE and layered on a 10-40% Sucrose gradient (in 5% increments of 5.5 ml each) in 0.5 M NaCl, 50 mM Tris pH8.0, 5 mM EDTA. Following centrifugation for 20 hours at 26,000 rpm in a SW28 rotor, the tubes were punctured and 1 ml fractions collected. Fifteen µl samples of each third fraction were run on 0.8 % agarose gel and the size of the DNA determined by comparison with linearized lambda DNA and *Hin*dIII-digested lambda DNA standards. Fractions containing DNA of 25-35 kb fragments were pooled, desalted on AMICON10 columns (7000 rpm; 20°C; 45 minutes) and concentrated by precipitation. This procedure yielded 50 ug of LBAA DNA of the required size.

Plasmid pHC79 (Hohn and Collins, 1980) DNA and a *Hin*dIII-phosphatase treated vector was prepared as described elsewhere (Maniatis et al., 1982). The ligation conditions were as follows:

Vector DNA (*Hind*III- and calf alkaline phosphatase-treated)	1.6 μg
Size fractionated LBAA *Hind*III fragments	3.75 μg
10X ligation buffer 250 mM Tris-HCl, pH 8.0; 100 mM $MgCl_2$; 100 mM Dithiothreitol; 2 mM Spermidine	2.2 μl
T4 DNA ligase (Boehringer-Mannheim) (400 units/ul)	1.0 μl

H_2O to 22.0 μl

18 hours at 16°C.

The ligated DNA (4 μl) was packaged into lambda phage particles (Stratagene; Gigapack Gold) using the manufacturer's procedure.

E. coli SR200 Mpu+, grown overnight in L-Broth (with maltose at 0.2%), was infected with 50 μl of the packaged DNA. Transformants were selected on MOPS complete agar plus ampicillin and with glyphosate at 0.2 mM as P source.

Aliquot samples were also plated on MOPS (Neidhardt et al., 1974) complete agar plus ampicillin containing Pi at 1mM to titer the packaged cosmids. Cosmid transformants were isolated on this latter medium at a rate of ~10^5 per μg/LBAA *Hind*III DNA after 2 days at 37°C. Colonies arose on the glyphosate-agar from day 3 until day 10 with a final rate of 1 per 200-300 cosmids. Plasmid DNA was prepared from

twenty one cosmid transformants from the glyphosate plates. These cosmids fell into at least two classes based on the *Hin*dIII restriction pattern of the plasmid DNA. In Class I, all the cosmids had cloned 6.4 and 4.2 kb *Hin*dIII restriction fragments in common and in Class II, a ~23 kbp fragment in common. Ten cosmids, representative of the diversity of the cloned fragments, were re-transformed into *E. coli* SR200 Mpu+ and the glyphosate utilization trait verified by selection for growth on MOPS complete agar plus ampicillin plus glyphosate plates. The final cell density achieved by the cultures using glyphosate (0.2mM in MOPS medium) as a P source was also determined and little difference could be discerned between the different transformants. Transformants were also inoculated into MOPS complete broth with AMPA at 0.1 mM as P source (to ensure the presence of "C-P lyase" activity) and after 24 hours at 37°C were diluted 100-fold into MOPS complete medium with glyphosate at 0.1 mM and [3-^{14}C]glyphosate (40,000 cpm/ml). All the cosmid-containing cells degraded glyphosate and generated N-acetylAMPA and N-acetylmethylamine, with no great difference in the rate. The N-acetylAMPA was found in the culture supernatant in these tests. One cosmid from Class I, identified as pMON7468, was chosen for further study. A second glyphosate oxidoreductase gene has been identified from a Class II cosmid clone.

Cell-free lysates *E. coli* SR200 Mpu+/pMON7468 were prepared from cells grown on MOPS complete medium with glyphosate at 1.0 mM (and supplemented with L-phenylalanine, L-tyrosine and L-tryptophan each at 100 μg/ml and with para-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid and para-aminobenzoic acid each at 5 μg/ml to minimize the effects of inhibition of the *E. coli* EPSP synthase). The cell pellet (approx. 0.5 g wet weight) was resuspended in 1 ml of lysis buffer (40 mM MOPS, pH7.4; 4 mM Tricine, pH 7.4;

10% glycerol; 1 mM DTT) and passed twice through a French Press. The cell debris was removed by centrifugation at 15000 rpm for 10 minutes. The supernatant was assayed, following addition of $MgCl_2$ to 10 mM, for degradation of radiolabeled glyphosate. The glyphosate substrate was supplied as [3-^{14}C]glyphosate (final concentration = 17 μM). The products observed were predominantly AMPA and some N–acetylAMPA; the production of AMPA is indicative of the cloned enzymatic activity from strain LBAA but the N–acetylAMPA could be due to endogenous *E. coli* activities (Avila et al., 1987). The specific activity for AMPA formation under these conditions was 13.3 pmoles AMPA/minute.mg protein.

Characterization of the Glyphosate-to-AMPA Gene

The cloned region responsible for this glyphosate oxidoreductase enzymatic activity was then localized in the cosmid. Deletions of pMON7468 were isolated, primarily within the cloned region, by using restriction enzymes that cut infrequently within the insert, as follows: plasmid DNA samples of 0.5 - 2 μg were digested to completion with restriction endonucleases *Not*I, *Sac*I, *Bgl*II or *Bam*HI, extracted with phenol:chloroform, ethanol precipitated, resuspended in TE buffer and ligated for 2-4 hours at room temperature (or for 18 hours at 16°C) in a final volume of 50 μl with ligation buffer and T4 DNA ligase. Transformants were selected in *E. coli* SR200 Mpu$^+$ and these deletions were examined for loss or retention of the glyphosate utilization phenotype. These data, in conjunction with restriction mapping of the clones, were used to localize the active region to near the central portion of the insert in pMON7468 that included the two common *Hin*dIII fragments (6.4 and 4.2 kb). The *Hin*dIII restriction fragments from this region were then

subcloned into pBlueScript (Stratagene) and their glyphosate phenotype determined in *E. coli* JM101 Mpu+ (the Mpu+ derivative of JM101 was isolated as described for SR200 Mpu+). Clones containing the 6.4 kb *Hin*dIII fragment, in either orientation, resulted in glyphosate utilization. Following restriction mapping of this *Hin*dIII fragment, a series of deletion clones were isolated from the two 6.4 kb *Hin*dIII clones using enzymes that cut infrequently in the insert and also in the polylinker region. A number of restriction fragments internal to the *Hin*dIII fragment were also subcloned. The 3.5 kb *Pst*I and 2.5 kb *Bgl*II fragments, in either orientation, were positive for glyphosate utilization. These data, combined with those from the deletions, were used to localize the active region to an approximately 1.8 kb *Bgl*II-*Xho*I fragment. In addition, deletions isolated from the 6.4 kb *Hin*dIII fragment indicated a minimum coding region size of around 0.7 kb, with the *Eco*RI and *Sac*I sites probably located within the coding sequences.

The direction of transcription/expression of the locus responsible for the glyphosate-to-AMPA enzymatic activity was determined as follows: *E. coli* JM101 Mpu+ transformants of pMON7469 #1 and #4 (Clones of the 2.5 kb *Bgl*II fragment in the BamHI site of pUC118; opposite orientations) were grown in M9-glucose-thiamine- ampicillin broth, with and without the *Plac* inducer IPTG, harvested in late log phase (Klett 190-220), cell-free lysates of the four cultures were prepared as described above and were assayed for glyphosate-to-AMPA activity with glyphosate at 17 μM. The highest enzymatic activity was obtained for pMON7469 #1 plus IPTG, where the *Xho*I site is distal to the *Plac*, suggesting that the gene(s) were expressed in the *Bgl*II-to-*Xho*I direction.

TABLE II - Glyphosate to AMPA Activity in Cell-Free Lysates of *E. coli* Transformants

Clone	IPTG added	Specific Activity nmoles AMPA /min.mg
pMON7469#1	no	< 3.0
pMON7469#1	yes	32.0
pMON7469#4	no	< 3.0
pMON7469#4	yes	< 3.0

The only product observed was AMPA, suggesting that the AMPA acetylating activity that was described earlier had been induced in *E. coli* transformants growing on glyphosate as the P source.

In a later experiment, cell lysates of pMON7469#1 and pMON7470 (*Bgl*II-*Xho*I 1.8 kb in pUC118; formed from pMON7469 #1 by deletion of the ~ 700 bp *Xho*I-*Sal*I fragment) were assayed for glyphosate-to-AMPA activity with glyphosate at 2 mM (Sp. Act. [3-^{14}C]glyphosate = 3.7 mCi/mmol; 0.2 µCi/reaction; cultures grown with IPTG in medium) and much higher enzymatic activities were recorded, reflecting the improved assay conditions.

TABLE III - Glyphosate to AMPA Activity in Cell-Free Lysates of *E. coli* Transformants

Clone	Specific Activity nmoles AMPA/min.mg
pMON7469#1	15.04
pMON7470	7.15

The proteins encoded by the *Bgl*II fragment were determined *in vivo* using a T7 expression system (Tabor and Richardson, 1985) following cloning of this fragment into the *Bam*HI site in the vector pBlueScript (+) (pMON7471 #1, #2; opposite orientations). Test and control plasmids were transformed into *E. coli* K38 containing pGP1-2 (Tabor and Richardson, 1985) and grown at 30°C in L-broth (2 ml) with ampicillin and kanamycin (100 and 50 μg/ml, respectively) to a Klett reading of ~ 50. An aliquot was removed and the cells collected by centrifugation, washed with M9 salts (Miller, 1972) and resuspended in 1 ml M9 medium containing glucose at 0.2%, thiamine at 20 μg/ml and containing the 18 amino acids at 0.01% (minus cysteine and methionine). Following incubation at 30°C for 90 minutes, the cultures were transferred to a 42°C water bath and held there for 15 minutes. Rifampicin (Sigma) was added to 200 μg/ml and the cultures held at 42°C for 10 additional minutes and then transferred to 30°C for 20 minutes. Samples were pulsed with 10 μCi of ^{35}S-methionine for 5 minutes at 30°C, the cells collected by centrifugation and suspended in 60-120 μl cracking buffer (60 mM Tris-HCl 6.8/1% SDS/1% 2-mercaptoethanol/10% glycerol/0.01% bromophenol blue). Aliquot samples were electrophoresed on 12.5% SDS-PAGE and following soaking for 60 minutes in 10 volumes of Acetic Acid-Methanol–water (10:30:60), the gel was soaked in ENLIGHTNING ™ (DUPONT) following manufacturer's directions, dried, and exposed at -70°C to X-Ray Film. Proteins labeled using ^{35}S-methionine were detected only for the *Bgl*II-to-*Xho*I direction, the largest about 45 kd in size. When the *Bgl*II-*Xho*I fragment was examined following cloning into the *Bam*HI-*Xho*I sites of pBlueScript (to form pMON7472), this ~45 kd protein was still expressed.

The effect of expression of the glyphosate-to-AMPA activity on glyphosate tolerance of *E. coli* was determined initially by examining the growth of recombinants in media containing inhibitory concentrations of glyphosate. The test compared the growth of *E. coli* JM101 containing a control vector (pUC118; Viera and Messing, 1987) or the pUC118 clones of the 2.5 kb *Bgl*II fragment (pMON7469 #1, #4). There was a very clear correlation between the glyphosate-utilization ability and glyphosate tolerance. This tolerance phenotype (resistance to 15 mM glyphosate) was then employed as a screen to quickly monitor for the phenotype of deletion clones such as pMON7470 (*Bgl*II-*Xho*I 1.8 kb in pUC118; formed from pMON7469 #1 by deletion of the ~ 700 bp *Xho*I-*Sal*I fragment) and later clones.

Nucleotide Sequence of the Structural Glyphosate Oxidoreductase Gene

The nucleotide sequence of the *Bgl*II-*Xho*I fragment (SEQ ID NO:3) was determined using single-stranded DNA templates (generated using the phagemid clones and the "helper" M13 phage R408) and the commercially available SEQUENASE ™ (International Biotechnologies, Inc.) kit. Computer analysis of the sequence (SEQ ID NO:3) revealed a single large open reading frame (ORF) in the *Bgl*II to *Xho*I direction and is presented in Figure 2 which includes the location of some of the relevant restriction sites. The putative stop codon (UAA) was located 2 bp 5' of the *Sca*I restriction cut site. Data to confirm that this UAA codon was the termination codon of the ~45 kd ORF were derived as follows: previously the 3' limits had been determined, based on the glyphosate utilization phenotype, to be between the *Sac*I site (95 bp upstream of the *Sca*I site) and the *Xho*I site. When the *Bgl*II-*Sca*I fragment was cloned into the *Bam*HI-*Sma*I sites of pBlueScript and the proteins expressed *in vivo*, the ~45 kd

protein was still produced. The *Bgl*II-*Sca*I fragment was then recloned from this pBlueScript clone as *Xba*I-*Hin*dIII into pUC118 *Xba*I-*Hin*dIII and was found to confer resistance to 15 mM glyphosate to *E. coli* JM101 transformants. These data located the C-terminus of the ~45 kd protein between the *Sac*I and *Sca*I sites. The only stop codon, in any reading frame, between these sites is that immediately upstream of the *Sca*I site.

There were two methionine codons (AUG; located at positions 120 and 186) that if used as the fMet would give rise to proteins of 46.140 and 44.002 kd, respectively, but neither was preceded by a clearly recognizable Shine-Dalgarno sequence.

The start of the protein was delineated more precisely as follows: *Bgl*II restriction site recognition sequences were introduced at positions upstream of the two potential start codons by site-directed mutagenesis of pMON7470, substituting AGATCT for the sequences AGACTG ("Bg120") and GTATGC ("Bg186"), 21 and 9 bp upstream of the AUG_{120} and AUG_{186}, respectively. Except where noted, oligonucleotide primers for mutagenesis comprised the sequences to be altered flanked by 8-10 homologous bases on each side. The glyphosate tolerance was determined for the mutated clones. Introduction of the *Bgl*II site upstream of AUG_{120} had no effect on glyphosate tolerance while it was abolished by the mutagenesis that introduced the *Bgl*II site upstream of AUG_{186}. The effects of these mutageneses on the ~ 45 kd protein were examined by subcloning the mutated sequences into T7 expression vectors using a site in the polylinker of pMON7470 (*Kpn*I), just upstream of the original *Bgl*II site, and the downstream *Hin*dIII site. This complete fragment was recloned into p18UT3T7 (PHARMACIA) *Kpn*I-*Hin*dIII and tested in vivo as described above. The ~ 45 kd protein was still expressed and at comparable levels from both of the "*Bgl*II" mutagenized

sequences. When the new *Bgl*II sites were used as 5' ends (and the downstream *Hin*dIII site) for cloning into the pBlueScript *Bam*HI-*Hin*dIII sites, the ~45 kd protein was still expressed when the new *Bgl*II site upstream of AUG_{120} served as 5' end, but not when that located upstream of AUG_{186} was the 5' end. These data suggest strongly that the AUG_{120} (or some codon located very close to it) is the N-terminus of the glyphosate oxido-reductase protein. The *Bgl*II site introduced upstream of the AUG_{186} did not result in a prematurely terminated or highly unstable protein and suggests that the predicted coding sequence changes resulting from this mutagenesis (Val_{18}-Cys_{19} --> Arg_{18}-Ala_{19}) had severe effects on the activity of the enzyme.

Further data to confirm the location of the N-terminus were obtained by introducing separately (by mutageneses of pMON7470), an *Nco*I restriction site recognition sequence (CCATGG for CTATGT; changes the second codon from Serine to Alanine) or an *Nde*I sequence (CATATG for CCTATG) at AUG_{120} and expressing this ORF using efficient *E. coli* expression vectors. The expression of the *Nde*I version is outlined here: the *Nde*I-*Hin*dIII fragment, beginning at the putative AUG, was cloned into pMON2123 (*Nde*I-*Hin*dIII) replacing the ompF-IGF-1 fusion fragment (Wong et al., 1988). The resultant clone was introduced into *E. coli* JM101 and the cells induced with nalidixic acid as described (Wong et al., 1988) for 2 hours. The resultant protein was indistinguishable in size from the ~45 kd protein on SDS PAGE and a cell lysate from an induced culture had a glyphosate oxidoreductase specific activity of 12.8 nmoles AMPA/min.mg. When compared in a separate experiment, no differences were observed for the glyphosate oxidoreductase activity when the second codon was Alanin instead of Serine. The structural DNA sequence for the glyphosate oxidoreductase

enzyme (SEQ ID NO:4) begins at nucleotide 120 and ends at nucleotide 1415 of the *Bgl*II-XhoI fragment of Figure 2 and the glyphosate oxidoreductese enzyme consists of 431 amino acids (SEQ ID NO:5).

Construction of Glyphosate Oxidoreductase Plant Gene Transformation Vectors

To facilitate the manipulation of the structural glyphosate oxidoreductase gene, the internal *Eco*RI and *Nco*I restriction site recognition sequences were removed by site-directed mutagenesis to substitute the sequence GAATTT for GAATTC and CCACGG for CCATGG, respectively. A glyphosate oxidoreductase coding sequence suitable for introduction into and expression in plant transformation vectors was assembled in the following way: the *Nco*I ("Met-Ala-") N-terminus was combined with the *Nco*I- and *Eco*RI-deleted coding sequences, and the C-terminus deleted to the *Sca*I site, in a number of cloning steps using the internal *Sph*I and *Eco*RV restriction sites. In these steps a *Bgl*II site was located immediately upstream of the *Nco*I site and *Eco*RI and *Hin*dIII sites were located immediately downstream from the stop codon. The sequence of this manipulated glyphosate oxidoreductase gene (SEQ ID NO:6) is shown in Figure 3. The manipulated glyphosate oxidoreductase gene still codes for the wild-type glyphosate oxidoreductase protein. The manipulations do not alter the amino acid sequence of the glyphosate oxidoreductase. This glyphosate oxidoreductase structural sequence (SEQ ID NO:6), as a *Bgl*II/*Nco*I--*Eco*RI/*Hin*dIII fragment of 1321 bp, is readily cloned into an appropriate plant expression cassette. This glyphosate oxidoreductase gene (SEQ ID NO:6) was cloned as a *Bgl*II-*Eco*RI fragment into the plant transformation and expression vector pMON979 to form pMON17073.

Modification and Resynthesis of the Glyphosate Oxidoreductase Gene Sequence

The glyphosate oxidoreductase gene from LBAA contains sequences that could be inimical to high expression of the gene in plants. These sequences include potential polyadenylation sites that are often A+T-rich, a higher G+C% than that frequently found in plant genes (56% *versus* ~50%), concentrated stretches of G and C residues, and codons that are not used frequently in plant genes. The high G+C% in the glyphosate oxidoreductase gene has a number of potential consequences including the following: a higher usage of G or C than that found in plant genes in the third position in codons, and the potential to form strong hair-pin structures that may affect expression or stability of the RNA. The reduction in the G+C content of the glyphosate oxidoreductase gene, the disruption of stretches of G's and C's, the elimination of potential polyadenylation sequences, and improvements in the codon usage to that used more frequently in plant genes, could result in higher expression of glyphosate oxidoreductase in plants.

In the first phase of this experiment, selected regions of the gene were modified by site-directed mutagenesis. These modifications were directed primarily (but not exclusively) at reducing the G+C% and at breaking up some of the G+C clusters. The manipulated glyphosate oxidoreductase gene was first recloned into the phagemid vector pMON7258 as a *Nco*I-*Hin*dIII fragment to form pMON17014. Single stranded DNA was prepared from a *dut ung E. coli* strain. Seven regions of the gene were modified by site-directed mutagenesis using the primers listed in Table IV and the Bio Rad mutagenesis kit (Catalog #170-3576) and following the protocols provided with this kit.

For the sake of clarity, the reverse complement of the actual primers is presented. The base positions, in the sequences presented in Figure 2 and in Figure 3, corresponding to the primers are indicated by the first and second set of numbers, respectively.

TABLE IV - Primers to Modify the Glyphosate Oxidoreductase Gene Coding Sequence

PRIMER 1 (149-210; 38-99)

CGCTGGAGCT GGAATCGTTG GTGTATGCAC TGCTTTGATG CTTCAACGTC GTGGATTCAA AG (SEQ ID NO:27)

PRIMER 2 (623-687; 512-576)

GCAGATCCTC TCTGCTGATG CTTTGCGTGA TTTCGATCCT AACTTGTCGC ATGCTTTTAC CAAGG (SEQ ID NO:28)

PRIMER 3 (792-832; 681-721)

GTCATCGGTT TTGAGACTGA AGGTCGTGCT CTCAAAGGCA T (SEQ ID NO:29)

PRIMER 4 (833-901; 722-790)

TACAACCACT AACGGTGTTC TGGCTGTTGA TGCAGCTGTT GTTGCAGCTG GTGCACACTC TAAATCACT (SEQ ID NO:30)

PRIMER 5 (1031-1091; 920-980)

GGAAATGGGT CTTCGTGTTG CTGGTACTGT TGAGTTTGCT GGTCTCACAG CTGCTCCTAA C (SEQ ID NO:31)

PRIMER 6 (1179-1246; 1068-1135)

TGGATGGGTT TTCGTCCTAG CATTCCTGAT TCTCTTCCAG TGATTGGTCG TGCAACTCGT ACACCCGA (SEQ ID NO:32)

PRIMER 7 (1247-1315; 1136-1204)
CGTAATCTAT GCTTTTGGTC ACGGTCATCT CGGTATGACA GGTGCTCCAA TGACTGCAAC TCTCGTCTC (SEQ ID NO:33)

The resultant gene (SEQ ID NO:7) was confirmed by sequencing and by the ability to provide comparable glyphosate tolerance levels as the manipulated glyphosate oxidoreductase gene control. This modified gene (SEQ ID NO:7) is referred to as "modified glyphosate oxidoreductase." The G+C% of the glyphosate oxidoreductase gene (SEQ ID NO:6) was reduced from ~56% in the manipulated version to ~52% in the modified version (SEQ ID NO:7). A comparison of the manipulated and modified glyphosate oxidoreductase gene is shown in Figure 3, with the manipulated version on top and the changes introduced to make the modified version on the bottom. This modified glyphosate oxidoreductase gene was cloned as a *Bgl*II-*Eco*RI fragment into a plant expression cassette comprising the En-CaMV35S promoter and the NOS 3' sequences. This cassette was then cloned as a NotI fragment into the pMON886 vector to form pMON17032 (Figure 5).

A synthetic glyphosate oxidoreductase gene (SEQ ID NO:8) was designed to change as completely as possible those inimical sequences discussed above. In summary, the gene sequence was redesigned to eliminate as much as possible the following sequences or sequence features (while avoiding the introduction of unnecessary restriction sites): stretches of G's and C's of 5 or greater; A+T rich regions (predominantly) that could function as polyadenylation sites or potential RNA destabilization region, and codons not frequently found in plant genes. A comparison of the manipulated (SEQ ID NO:6) and synthetic (SEQ ID NO:8) glyphosate oxidoreductase genes is presented in Figure 4, with the manipulated gen (SEQ ID NO:6) on top and the differences introduced into the synthetic

gene (SEQ ID NO:8) on the bottom. The G+C% for the synthetic glyphosate oxidoreductase gene is ~51% and the potential to form short, high energy, hair-pin structures is reduced. This synthetic gene was cloned as a *Bgl*II-*Eco*RI fragment into pMON979 to form pMON17065 for introduction into plants.

Expression of Chloroplast Directed Glyphosate Oxidoreductase

The glyphosate target in plants, the 5-*enol*pyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme, is located in the chloroplast. Although glyphosate oxidoreductase activity located in the cytoplasm reduces/prevents glyphosate from reaching the chloroplast in the transgenic plant, directing the glyphosate oxidoreductase enzyme to the chloroplast has been found to further minimize the effects of glyphosate on EPSP synthase. Many chloroplast-localized proteins are expressed from nuclear genes as precursors and are targeted to the chloroplast by a chloroplast transit peptide (CTP) that is removed during the import steps. Examples of such chloroplast proteins include the small subunit (SSU) of Ribulose-1,5-bisphosphate carboxylase (RUBISCO), 5–*enol*-pyruvyl-shikimate-3-phosphate synthase (EPSPS), Ferredoxin, Ferredoxin oxidoreductase, the Light-harvesting-complex protein I and protein II, and Thioredoxin F. It has been demonstrated *in vivo* and *in vitro* that non-chloroplast proteins may be targeted to the chloroplast by use of protein fusions with a CTP and that a CTP sequence is sufficient to target a protein to the chloroplast (della-Cioppa et al., 1987).

The glyphosate oxidoreductase protein was targeted to the chloroplast by construction of a fusion between the C–terminus of a CTP and the N–terminus of glyphosate oxidoreductase. In the first example, a specialized CTP, derived from the SSU 1A gene from *Arabidopsis thaliana* (Timko et al., 1988) was used. This CTP (designated CTP1) was

constructed by a combination of site-directed mutageneses. The CTP1 structure (SEQ ID NO:9) (Figure 6) is made up of the SSU 1A CTP (amino acids 1-55), the first 23 amino acids of the mature SSU 1A protein (amino acids 56-78), a serine residue (amino acid 79), a new segment that repeats amino acids 50 to 56 from the SSU 1A CTP and the first two amino acids from the mature protein (amino acids 80-87), and an alanine and methionine residue (amino acids 88 and 89). An *Nco*I restriction site is located at the 3' end (spans the Met codon) to facilitate the construction of precise fusions to the 5' of glyphosate oxidoreductase or other genes. At a later stage, a *Bgl*II site was introduced upstream of the N terminus of the SSU 1A sequences to facilitate the introduction of the fusions into plant transformation vectors. A fusion was assembled between the CTP1 (SEQ ID NO:9) and the manipulated glyphosate oxidoreductase (SEQ ID NO:6) (through the *Nco*I site) in the pGEM3zf(+) vector to form pMON17034. This vector may be transcribed *in vitro* using the SP6 polymerase and the RNA translated with ^{35}S-Methionine to provide material that may be evaluated for import into chloroplasts isolated from *Lactuca sativa* using the methods described hereinafter (della-Cioppa et al., 1986, 1987). This CTP1-glyphosate oxidoreductase fusion was indeed found to be imported into chloroplasts at about 9% efficiency of that of the control, ^{35}S labeled PreEPSPS (pMON6140; della-Cioppa et al., 1986). A CTP1-glyphosate oxidoreductase fusion was then assembled with the synthetic glyphosate oxidoreductase gene (SEQ ID NO:8) and this was introduced as a *Bgl*II-*Eco*RI fragment into plant vector pMON979 to form pMON17066 (Figure 7). Following an intermediate cloning step to acquire more cloning sites, this CTP1-glyphosate oxidoreductase fusion was also cloned as a *Xba*I-*Bam*HI site into pMON981 to form pMON17138 (Figure 8).

In the second example, a CTP-glyphosate oxidoreductase fusion was constructed between the *Arabidopsis thaliana* EPSPS (Klee et al., 1987) CTP and the synthetic glyphosate oxidoreductase coding sequences. The *Arabidopsis* CTP was first engineered by site-directed mutagenesis to place a *Sph*I restriction site at the CTP processing site. This mutagenesis replaced the Glu-Lys at this location with Cys-Met. The sequence of this CTP, designated CTP2, (SEQ ID NO:10) is shown in Figure 9. The *Nco*I site of the synthetic glyphosate oxidoreductase gene (SEQ ID NO:8) was replaced with a *Sph*I site that spans the Met codon. The second codon was converted to one for leucine in this step also. This change had no apparent effect on the *in vivo* activity of glyphosate oxidoreductase in *E. coli*. The CTP2-synthetic glyphosate oxidoreductase fusion was cloned into pBlueScript KS(+) and this template was transcribed *in vitro* using T7 polymerase and the ^{35}S-methionine-labeled material was shown to import into chloroplasts with an efficiency comparable to that for the CTP1-glyphosate oxidoreductase fusion. This CTP2-synthetic glyphosate oxidoreductase fusion was then cloned as a *Xba*I-*Bam*HI fragment into a plant expression vector to form pMON17164. A structural map of this plasmid is presented in Figure 12.

The plant vector portion of pMON17164 (Figure 12) is composed of the following segments. A chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 Kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 Kb neomycin phosphotransferase typeII gene (KAN), and the 0.26Kb 3'-non-translated region of the nopalin e synthase gene (NOS 3') (Fraley et al., 1983). A 0.45 Kb ClaI to DraI fragment from the pTi15955 octopine Ti plasmid, which contains the T-DNA left border region (Barker

et al., 1983) A 0.75 Kb segment containing the origin of replication from the RK2 plasmid (ori-V) (Stalker et al., 1981) A 3.0 Kb SalI to PstI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322) and the bom site for the conjugational transfer into *Agrobacterium tumefaciens* cells. A 0.93 Kb fragment isolated from transposon Tn7 which encodes bacterial spectinomycin/streptomycin resistance (Spc/Str) (Fling et al., 1985), and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*. A 0.36 Kb PvuI to BclI fragment from the pTiT37 plasmid, which contains the nopaline-type T-DNA right border region (Fraley et al., 1985). An expression cassette consisting of the 0.6 Kb 35S promoter from the figwort mosaic virus (P-FMV) (Gowda et al., 1989), several unique cloning sites, and the 0.7 Kb 3' nontranslated region of the pea rbcS-E9 gene (E9 3') (Coruzzi et al., 1984, and Morelli et al., 1985). The CTP2-synthetic glyphosate oxidoreductase fusion fragment was cloned into this expression cassette. The introduction of this plasmid into *Agrobacterium* and subsequent plant transformation is described in the Examples to follow.

Those skilled in the art will recognize that various chimeric constructs can be made which utilize the functionality of a particular CTP to import the contiguous glyphosate oxidoreductase enzyme into the plant cell chloroplast. The chloroplast import of the glyphosate oxidoreductase can be determined using the following assay.

Chloroplast Uptake Assay

Intact chloroplasts are isolated from lettuce (*Latuca sativa*, var. longifolia) by centrifugation in Percoll/ficoll gradients as modified from Bartlett et al. (1982). The final pellet of intact chloroplasts is suspended in 0.5 ml of sterile 330 mM

sorbitol in 50 mM Hepes-KOH, pH 7.7, assayed for chlorophyll (Arnon, 1949), and adjusted to the final chlorophyll concentration of 4 mg/ml (using sorbitol/Hepes). The yield of intact chloroplasts from a single head of lettuce is 3-6mg chlorophyll.

A typical 300 µl uptake experiment contained 5 mM ATP, 8.3 mM unlabeled methionine, 322 mM sorbitol, 58.3 mM Hepes-KOH (pH 8.0), 50 µl reticulocyte lysate translation products, and intact chloroplasts from *L. sativa* (200 µg chlorophyll). The uptake mixture is gently rocked at room temperature (in 10 x 75 mm glass tubes) directly in front of a fiber optic illuminator set at maximum light intensity (150 Watt bulb). Aliquot samples of the uptake mix (about 50 µl) are removed at various times and fractionated over 100 µl silicone-oil gradients (in 150 µl polyethylene tubes) by centrifugation at 11,000 X g for 30 seconds. Under these conditions, the intact chloroplasts form a pellet under the silicone-oil layer and the incubation medium (containing the reticulocyte lysate) floats on the surface. After centrifugation, the silicone-oil gradients are immediately frozen in dry ice. The chloroplast pellet is then resuspended in 50-100 µl of lysis buffer (10 mM Hepes-KOH pH 7.5, 1 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-n-caproic acid, and 30 µg/ml aprotinin) and centrifuged at 15,000 X g for 20 minutes to pellet the thylakoid membranes. The clear supernatant (stromal proteins) from this spin, and an aliquot of the reticulocyte lysate incubation medium from each uptake experiment, are mixed with an equal volume of 2X SDS-PAGE sample buffer for electrophoresis (see below).

SDS-PAGE is carried out according to Laemmli (1970) in 3-17% (w/v) acrylamide slab gels (60 mm X 1.5 mm) with 3% (w/v) acrylamide stacking gels (5 mm X 1.5 mm). The gel is fixed for 20-30 minutes in a solution with 40% methanol and 10% acetic acid. Then, the gel is soaked in EN³HANCE™

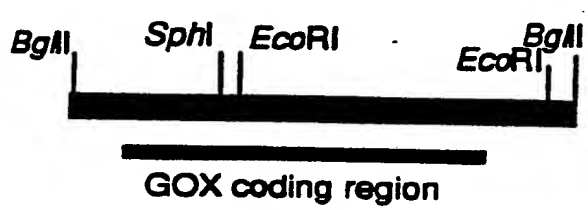
(DuPont) for 20-30 minutes, followed by drying the gel on a gel dryer. The gel is imaged by autoradiography, using an intensifying screen and an overnight exposure to determine whether the glyphosate oxidoreductase is imported into the isolated chloroplasts.

Alternative Isolation Protocol for Other Glyphosate Oxidoreductase Structural Genes

A number of other glyphosate oxidoreductase genes have been identified and cloned, including the second LBAA glyphosate oxidoreductase gene from the Class II cosmid pMON7477. The gene was located, by Southern hybridization, on the ~23 kb *Hin*dIII fragment, discussed in the cloning section above, using the first glyphosate oxidoreductase gene as a probe. Southern analysis also showed *Pst*I and *Bgl*II hybridizing bands of ~3.5 and ~2.5 kb, respectively. The *Bgl*II fragment from pMON7477 was subcloned into the *Bam*HI site of pBlueScript vector. A clone in *E. coli* JM101 (pMON7482), in which the cloned fragment was oriented relative to the *lac* promoter as in pMON7469#1, was induced with IPTG and assayed for glyphosate oxidoreductase activity. In this experiment a Sp. Act. of ~93 nmol/min.mg was obtained. In a later experiment, Class I and Class II cosmids were also isolated following infection of *E. coli* JM101 with the same packaged cosmid preparation and selection directly for glyphosate tolerance at 3-5 mM glyphosate on M9 media.

A glyphosate oxidoreductase gene has also been subcloned from another microbial isolate, identified originally by its ability to utilize glyphosate as a phosphorous source and later shown to contain a putative glyphosate oxidoreductase gene by hybridization with the LBAA glyphosate oxidoreductase gene probe. This gene was cloned initially in a T7 promoter cosmid by screening for glyphosate tolerance in *E. coli*

HB101/pGP1-2 (Boyer and Rolland-Dussoix, 1969; Tabor and Richardson, 1985) on M9 medium containing glyphosate at 3 mM. The presence of the glyphosate oxidoreductase gene was first indicated by a positive hybridization signal with the LBAA gene and by its location on a 2.5 kb *Bgl*II fragment. This *Bgl*II fragment was cloned into the *Bam*HI site in pBlueScript (pMON17183) and expressed from the *lac* promoter by addition of IPTG. In this experiment a glyphosate oxidoreductase with a specific activity of 53 nmoles/min.mg was obtained, confirming the isolation of the gene by this strategy. The following features have usually been found for these glyphosate oxidoreductase genes: the genes are found (by Southern hybridization using full-length glyphosate oxidoreductase gene probes) on ~2.5 kb *Bgl*II fragments, on ~3.5 *Pst*I fragments, contain one *Eco*RI site within the gene and the genes do not contain a *Hin*dIII site. The following schematic diagram illustrates some common features of these genes.



The high degree of similarity of glyphosate oxidoreductase genes also suggests another way by which new glyphosate oxidoreductase genes may be cloned. The apparent conservation of regions flanking the genes and the absence of certain restriction sites suggests the use of single-stranded oligonucleotide probes to the flanking regions, containing restriction sites for *Bgl*II, *Hin*dIII, *Pst*I, *Bam*HI, *Nde*I, or other suitable cloning sites, and PCR (Polymerase Chain Reaction; see Erlich, 1989, for complete details on PCR and its applications) to amplify a glyphosate oxidoreductase gene fragment suitable for cloning. The flanking sequences for 119

bp upstream (SEQ ID NO:11) of the wild-type (LBAA isolate) glyphosate oxidoreductase gene and for ~290 bp (SEQ ID NO:12) downstream of the gene are provided in Figure 2.

Using this PCR approach, glyphosate oxidoreductase genes from a number of sources have been isolated. The presence of the glyphosate oxidoreductase activity was confirmed by cloning the glyphosate oxidoreductase gene from chromosomal DNA prepared from *Pseudomonas* sp. strain LBr (Jacob et al., 1988) and using primers homologous to the N- and C-termini of the LBAA glyphosate oxidoreductase gene and containing the following suitable restriction cloning sites: 5'-GAGAGACTGT CGACTCCGCG GGAGCATCAT ATG-3' (SEQ ID NO:13) and 5'-GAACGAATCC AAGCTTCTCA CGACCGCGTA AGTAC-3' (SEQ ID NO:14). Cyclotherm parameters used for these PCR reactions is as follows:

Denature at 94° C for 1 minute;
Anneal at 60° C for 2 minutes;
Polymerize at 72° C for 3 minutes,
30 cycles, no autoextension, linked to 4° C incubation.

The expected ~1.3 kb PCR produced was generated and following digestion with *Nde*I and *Hind*III, this fragment was cloned into pMON2123 for expression of the encoded enzyme. The glyphosate oxidoreductase activity was measured as described above and the K_m for glyphosate was similar to that for enzymes from LBAA which is presented supra.

source of glyphosate oxidoreductase gene	K_m(glyphosate; mM)
Pseudomonas sp. strain LBr	25

Bacteria isolated from glyphosate process waste stream tr-atment facilities may also be capable of converting glyphosate to AMPA. *Pseudomonas* strains LBAA and LBr are

two such examples. Such bacteria may also be isolated *de novo* from these waste treatment facilities.

A population of bacteria was isolated from a fixed-bed immobilized cell column, which employed Mannville R-635 diatomaceous earth beads, by plating on Tryptone Soy Agar (Difco), containing cycloheximide at 100 ug/ml, and incubating at 28°C. The column had been run for three months on a waste-water feed from the Monsanto Company's Luling, MS, glyphosate production plant. The column contained 50 mg/ml glyphosate and NH_3 as NH_4Cl. Total organic carbon was 300 mg/ml and BOD's (Biological Oxygen Demand - a measure of "soft" carbon availability) was less than 30 mg/ml. This treatment column has been described (Heitkamp et al., 1990). One of the predominant members of this population, identified as *Agrobacterium* sp. strain T10, was found to also grow in minimal broth in which the sole carbon source provided was glyphosate at 10 mM (this broth was made up as for DF medium but with glyphosate substituting for the glucose, gluconate and citrate). Chromosomal DNA was prepared from this isolate and subjected to the same PCR procedure and with the same primers as described above for the strain LBr. A fragment of the correct size was generated and cloned into the *E. coli* expression vector. The glyphosate oxidoreductase activity was assayed and the K_m for glyphosate also determined:

source of gene	K_m(glyphosate: mM)
Agrobacterium sp. strain T10	28

Glyphosate-to-AMPA conversion has been reported for many different soils (see Malik et al., 1989 for a review) and a number of procedures are available for the extraction of total DNA from mixed environm nt samples such as soil (Holben et al., 1988; Steffan and Atlas, 1988; Tsai and Olson, 1991),

indicating the possibility of cloning glyphosate oxidoreductase genes without having to first isolate such a degrading microorganism. Of course, the procedure described for the cloning of the glyphosate oxidoreductase genes, based on the conferring of a glyphosate utilization ability or glyphosate tolerance on *E. coli*, provides a scheme by which other glyphosate oxidoreductase genes and other glyphosate metabolizing genes may be cloned, without relying on the homology determined for the glyphosate oxidoreductase gene described here. It is possible also to enrich for glyphosate degrading bacteria, for example, by the repeated application of glyphosate to a patch of soil (Quinn et al., 1988, Talbot et al., 1984). This enrichment step might be used to increase the ease with which glyphosate oxidoreductase genes are recovered from soil or other environments.

Evidence for the presence of the glyphosate oxidoreductase gene in soil bacteria and a procedure for the isolation of such genes is outlined in the following: A population of suitable bacteria was enriched for selection of bacteria capable of growing in liquid media with glyphosate (at 10 mM) as a source of carbon (This medium is made up as described for the Dworkin-Foster medium but with the omission of the carbon sources and with Pi as a source of P). The inoculum was provided by extracting soil (from a recently harvested soybean field in Jerseyville, Illinois) and the population selected by successive culturing in the medium described above at 28°C (cycloheximide was included at 100 μg/ml to prevent growth of fungi). Upon plating on L-agar medium, 5 colony types were identified. Chromosomal DNA was prepared from 2 ml L-broth cultures of these isolates and the presence of the glyphosate oxidoreductase gene was probed using PCR screening. Using the primers GCCGAGATGACCGTGGCCGAAAGC (SEQ ID NO:15) and

GGGAATGCCGGATGCTTCAACGGC (SEQ ID NO:16), a DNA fragment of the predicted size was obtained with the chromosomal DNA from one of the isolates (designated S3). The PCR conditions used were as follows: 1 minute at 94°C; 2 minutes at 40°C; 3 minutes at 72°C; 35 cycles. The DNA fragment generated in this way is used as a probe (following radiolabeling) to isolate the S3 glyphosate oxidoreductase gene candidate from a cosmid bank constructed as described for LBAA DNA and greatly facilitates the isolation of other glyphosate oxidoreductase genes. The primers used are homologous to internal sequences in the LBAA glyphosate oxidoreductase gene. The PCR conditions employed allow a fair degree of mismatch in the primers and the result suggests that the glyphosate oxidoreductase gene from S3 may not be as closely related to the other glyphosate oxidoreductase genes that were successfully isolated using the primers to the N- and C-termini of the LBAA gene.

A variety of procedures are available for the isolation of genes. Some of these procedures are based on the knowledge of gene function that allow the design of phenotypic screens to aid in the isolation. Others are based on at least partial DNA sequence information that allow the use of probes or primers with partial or complete homology, or are based on the use of antibodies that detect the gene product. All of these options may be applied to the cloning of glyphosate oxidoreductase genes.

Improvement of the Kinetic Properties of Glyphosate Oxidoreductase

Prior examples of engineered herbicide resistance by enzymatic inactivation of the herbicide have utilized enzymes with an ability to bind and metabolize the h rbicides much more efficiently than glyphosate oxidoreductase metabolizes

glyphosate. The glyphosate oxidoreductase enzyme has a K_m for glyphosate of 20–30 mM and, as a result, the reaction rate for the degradation of glyphosate may be enhanced for optimal efficiency in transgenic plants by either lowering the K_m or by raising the V_{max}.

Random mutagenesis techniques coupled with appropriate selections and/or screens are powerful tools which have been used successfully to generate large numbers of mutagenized gene sequences and potential variants. The same approaches may be used to isolate and to identify glyphosate oxidoreductase variants with improved glyphosate degradation efficiency. The mutagenesis techniques that may be employed include chemical mutagenesis of bacterial cultures containing the gene of interest or of purified DNA containing this gene and PCR methods used to generate copies of the gene (or portions of it) under conditions that favor misincorporation of nucleotides (errors) into the new strand. An example of such a condition would be carrying out the PCR reaction in the presence of Mn^{++}.

Appropriate *in vivo* screens for improved variants following the mutagenesis could include those for improved glyphosate tolerance in *E. coli* or increased growth on glyphosate in Mpu+ strains. For the screen, the glyphosate oxidoreductase gene is cloned into a vector containing a weak bacterial promoter and/or in a replicon with a low copy number. The glyphosate tolerance phenotypes of different glyphosate oxidoreductase constructs have been shown to vary over a range of glyphosate concentrations and to correlate with the level of glyphosate oxidoreductase expression. For example, under uninduced conditions, P*lac*-glyphosate oxidoreductase vectors express less glyphosate oxidoreductase than P*recA*-glyphosate oxidoreductase vectors and also display lower glyphosate tolerance. The mutagenized gene fragment is cloned into the most suitable vector and the resultant library

screened. Variants are selected for their ability to grow at glyphosate levels which inhibit growth of the control strain containing the parent glyphosate oxidoreductase clone. Glyphosate oxidoreductase activity confers on *E. coli* the ability to convert glyphosate to AMPA and, in suitable *E. coli* strains, this AMPA can provide a source of phosphate following cleavage of the C-P bond by C-P lyase. Suitable *E. coli* strains are B strains or Mpu+ derivatives of K strains. The glyphosate oxidoreductase gene confers minimal growth on glyphosate as the sole phosphorus source in strain *E. coli* JM101 Mpu+ (= GB993). The growth rate on glyphosate has been shown to also correlate with the glyphosate oxidoreductase expression level. The mutagenized glyphosate oxidoreductase gene is cloned into the appropriate vector and the variant library screened by differential growth rates on plates or by culturing in media containing glyphosate as sole phosphorous source. Clones which demonstrate faster growth on plates relative to the control strain are subsequently re-screened by growth curve analysis.

Glyphosate oxidoreductase variants which have been identified in each selection/screen are cloned into a vector for high-level expression and subjected to enzyme analysis to determine K_m and V_{max} values for glyphosate. The best glyphosate oxidoreductase variants are purified for complete kinetic characterization. Glyphosate oxidoreductase variants which have been identified with lower K_m values and similar or higher V_{max} values than wild-type enzyme values are analyzed by nucleic acid sequencing to determine the mutation(s). The goal in isolating variants would be to increase the k_{cat}/K_m ratio for glyphosate oxidoreductase-catalyzed glyphosate degradation.

A variant with such improvements was isolated. The mutagenesis procedure used was that of Mn^{++}-poisoned

PCR and the template was a linearized glyphosate oxidoreductase gene plasmid containing the synthetic glyphosate oxidoreductase gene (SEQ ID NO:8). The oligonucleotide primers used were homologous to regions in the vector and flanking the glyphosate oxidoreductase gene. The PCR conditions employed were as follows: 1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C and with 35 cycles. A 5:1 ratio of dCTP+dGTP+TTP to dATP was used. The reactions contained $MnCl_2$ at 125, 250, 375, or 500 µM. After the reaction, the amplified product was recloned into a vector containing a weak *E. coli* promoter. This vector was a pBR327 derivative containing the *araBAD* promoter and suitable cloning sites. One hundred colonies from this cloning step were then screened in *E. coli* GB993 for improved glyphosate tolerance and utilization phenotypes in media composed of MOPS minimal medium with glyphosate and Pi or with glyphosate alone, respectively. Growth rates were determined by measuring A_{550} over a 96 hour period. Three clones were identified that exhibited faster growth rates in these screens. These transformants had a 1.5-2.0-fold faster utilization phenotype. The glyphosate oxidoreductase gene was recloned into the expression vector portion and this phenotype verified. All kinetic analysis was performed on crude *E.coli* lysates. Putative glyphosate oxidoreductase variant proteins were overexpressed after subcloning the *NcoI/Hin*dIII variant glyphosate oxidoreductase gene into P*recA-gene10*L expression vector. For overexpression in P*recA-gene10*L constructs, GB993 cells containing the vector were induced at a Klett=110-120 in M9 minimal medium with 50 µg/ml nalidixic acid and allowed to grow for 2.5 hours at 37°C with vigorous shaking. Cells were harvested by centrifugation at 4000g, 5 minutes at 4°C, and resuspend in 100 mM Tris-HCl, pH 7.1, 1 mM EDTA, 35 mM KCl, 20% glycerol, and 1 mM benzamidine at 3ml/g cell pellet.

Lysates were prepared by breaking the cells in a French press, twice, at 1000 psi. Insoluble debris was removed by centrifugation at 12000g, 15 minutes at 4°C, and the supernatant was de-salted by passing over a PD-10 column (Sephadex G-25, Pharmacia). The void volume fraction was used as the source of enzyme for kinetic analysis. Protein concentrations were determined using the Bio-Rad protein dye-binding assay. Time and enzyme concentration courses were performed to determine linear ranges. The enzyme assay was performed as follows: lysate and glyphosate oxidoreductase mix (final concentration = 0.1 M MOPS, 0.01 M Tricine, pH 7.4, 0.01 mM FAD, 10 mM $MgCl_2$) in a 100 µl reaction were pre-incubated at 30°C for 2 minutes prior to the addition of glyphosate (analytical grade stock prepared in water adjusted to pH 7.0 with NaOH). Ten minutes was determined to be the optimal time for the enzyme assay using 10 µg lysate. After 10 minutes at 30°C with shaking, 0.25 ml dinitophenylhydrazine (DNPH) reagent (0.5 mg/ml in 0.5 M HCl) was added and the reaction was allowed to proceed for an additional 5 minutes at 30°C with shaking. A 1.5 M NaOH solution (400µl) was then added to the assay mix, and the reaction was continued for 5 minutes at 30°C with shaking. Enzyme activity was determined from the amount of glyoxylate-DNPH adduct formed by measuring A_{520} against a standard of glyoxylate. Enzyme assays are performed in duplicate on at least two different single colony isolates of a putative glyphosate oxidoreductase variant. To determine K_m and V_{max}, enzyme assays were performed over a (0.2-2.0) x K_m range of glyphosate concentrations. The K_m and V_{max} were determined from Lineweaver Burk, Eadie-Hofstee and hyperbolic kinetic plots. V_{max} was estimated after determining the amount of immunoreactive glyphosate oxidoreductase protein in lysates by

immunoblot analysis as d scribed below. Immunoblot analysis was performed following SDS-PAGE and transfer of protein from the gel to nitrocellulose at 500 mA in a Hoeffer transfer apparatus in 25 mM Tris-HCl, 192 mM glycine containing 0.1% SDS and 25% methanol for 1-2 hours. After transfer, the nitrocellulose was incubated with 50 mM Tris-HCl, pH7.5, 0.9% NaCl, 0.01% Tween 20, 0.02% NaN_3 containing 2% bovine serum albumin at room temperature with shaking for at least 30 minutes. After blocking, the same buffer containing a 1:25,000 dilution of goat anti-glyphosate oxidoreductase antiserum was added and the filter was allowed to shake at room temperature for 45 minutes. After incubation with primary glyphosate oxidoreductase antibody, the filter was washed for 45 glyphosate oxidoreductase minutes in buffer without antibody; buffer containing a 1:5000 dilution of rabbit anti-goat alkaline phosphatase-conjugated second antibody (from Pierce) was added and the filter was incubated for 45 minutes at room temperature with shaking. The filter was then washed in buffer without antibody for 30 minutes prior to addition of NBT and BCIP (Promega) to allow color development. Immunoreactive glyphosate oxidoreductase protein was also quantitated by dot blotting the lysate onto nitrocellulose and then processing the filter as described above, except that ^{125}I-Protein G was used for detection. The amount of glyphosate oxidoreductase protein in lysates was determined by counting the dot and comparing the amount of radioactivity against a glyphosate oxidoreductase protein standard. One variant, v.247, showed a 3-4-fold higher specific activity for glyphosate oxidoreductase at 25 mM glyphosate and the immunoblot analysis indicated that this was not due to an elevated glyphosate oxidoreductase protein level. Subsequent assays indicated that this variant had a 10-fold lower K_m for glyphosate than the wild type glyphosate oxidoreductase. In a

similar manner the K_m for IDA was also determined and these data are presented below.

Kinetic analysis of glyphosate oxidoreductase variants:

	app K_m (mM)		app V_m (U/mg)		V_m/K_m	
Variant	Glyp	IDA	Glyp	IDA	Glyp	IDA
wild type	27.0	2.8	0.8	0.5	.03	.18
v.247	2.6	0.7	0.6	0.7	.23	1.0

The glyphosate oxidoreductase gene from v.247 was sequenced (SEQ ID NO:17) and five nucleotide changes were found. These changes are described in the following as they relate to the codons: GCT to GCC (codon 43), no amino acid change; AGC to GGC (codon 84), Ser to Gly; AAG to AGG (codon 153), Lys to Arg; CAC to CGC (codon 334), His to Arg, and CCA to CCG (codon 362), no amino acid change. The amino acid sequence of the glyphosate oxidoreductase gene from v.247 is presented as SEQ ID NO:18. The importance of these different amino acid changes was determined initially by recloning the altered regions into wild type glyphosate oxidoreductase and determining the effect on glyphosate oxidoreductase activity and kinetics. This was accomplished by recloning the *Nco*I-*Nhe*I fragment (contains codon 84), the *Nhe*I-*Apa*LI fragment (contains codon 153), and the *Apa*LI-*Hind*III fragment (contains codon 334), seperately into the wild type gene. These glyphosate oxidoreductase genes were then expressed and the kinetic analyses performed. The data are presented below and indicate that the change that occured in the *Apa*LI-*Hind*III fragment (contains codon 334) was responsible solely for the alteration in the enzyme.

Kinetic analysis of domain switches

Clone	app K_m (mM)	app V_m (U/mg)	V_m/K_m
wt (w1w2w3*)	28.4	0.65	0.022
v.247(v1v2v3**)	2.1	0.72	0.34
w1v2w3	23.5	0.62	0.026
w1v2v3	2.1	0.6	0.28
w1w2v3	2.0	0.75	0.375
v1w2v3	2.6	0.55	0. 21
v1w2w3	28.0	0.75	0.027
v1v2w3	26.7	0.55	0.021

* w1=SER84; w2=LYS153; w3=HIS334

** v1=GLY84; v2=ARG153; v3=ARG334

This result was confirmed and extended by repeating the His to Arg change at codon 334 and introducing other specific changes at this residue by site-directed mutageneses. The primers used are listed in the following: Arg - CGTTCTCTAC ACTCGTGCTC GTAAGTTGC (SEQ ID NO:19); Lys - CGTTCTCTAC ACTAAGGCTC GTAAGTTGC (SEQ ID NO:20); Gln - CGTTCTCTAC ACTCAAGCTC GTAAGTTGC (SEQ ID NO:21); and Ala - CGTTCTCTAC ACTGCTGCTC GTAAGTTGC (SEQ ID NO:22) (These sequences are the antisense to those actually used). The presence of these changes was confirmed by sequencing the mutagenized glyphosate oxidoreductase genes and a kinetic analysis of the expressed glyphosate oxidoreductase enzymes was performed. The data are presented in the following and show that a number of substitutions are possible at this position and which result in an enzyme with altered kinetic properties.

Kinetic analysis of glyphosate oxidoreductase variants:

	app K_m (mM)		app V_m (U/mg)		V_m/K_m	
Variant	Glyp	IDA	Glyp	IDA	Glyp	IDA
wild type	27.0	2.8	0.8	0.5	.03	.18
v.247	2.6	0.7	0.6	0.7	.23	1.0
ARG 334	2.6	0.5	0.6	0.6	.23	1.2
LYS 334	9.9	1.3	0.7	0.8	.07	.62
GLN 334	19.6	3.5	0.6	0.7	.03	.20
ALA 334	26.7	3.5	0.2	0.2	.007	.057

Additonal mutageneses were performed to change the His334 residue to other amino acids. The primers to accomplish this and the new codon are listed in the following:

Trp - CTCTACACTTGGGCTCGTAAGCTTCTTCCAGC (SEQ ID NO:23);
Ile - CTCTACACTATCGCTCGTAAGCTTCTTCCAGC (SEQ ID NO:24);
Leu - CTCTACACTCTGGCTCGTAAGCTTCTTCCAGC (SEQ ID NO:25); and
Glu - CTCTACACTGAAGCTCGTAAGCTTCTTCCAGC (SEQ ID NO:26)

(These sequences are the antisense of those actually used; these primers also add a "silent" *Hin*dIII that facilitates the identification of the mutagenized progeny from the population). The GLU334 variant retains substantial glyphosate oxidoreductase activity, while the TRP334, ILE334, and LEU334 variants retain much less activity.

From the first generation variants, those with the highest k_{cat}/K_m ratio are preferably subjected to a second round of mutagenesis followed by subsequent screening and analysis. An alternative approach would be to construct second generation glyphosate oxidoreductase variants by combining single point mutations identified in the first generation variants.

PLANT TRANSFORMATION

Plants which can be made glyphosate tolerant by practice of the present invention include, but are not limited to, soybean, cotton, corn, canola, oil seed rape, flax, sugarbeet, sunflower, potato, tobacco, tomato, wheat, rice, alfalfa, lettuce, apple, poplar and pine.

A double-stranded DNA molecule of the present invention ("chimeric gene") can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella (1983), Bevan (1984), Klee (1985) and EPO publication 120,516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using viruses or pollen.

The pMON979 plant transformation/expression vector was derived from pMON886 (described below) by replacing the neomycin phosphotransferase typeII (KAN) gene in pMON886 with the 0.89 kb fragment containing the bacterial gentamicin-3-N-acetyltransferase type III (AAC(3)-III) gene (Hayford et al., 1988). The chimeric P-35S/AA(3)-III/NOS 3' gene encodes gentamicin resistance which permits selection of transformed plant cells. pMON979 also contains a 0.95 kb expression cassette consisting of the enhanced CaMV 35S promoter (Kay et al., 1987), several unique restriction sites, and the NOS 3' end (P-En-CaMV35S/NOS 3'). The rest of the pMON979 DNA segments are exactly the same as in pMON886.

Plasmid pMON886 is made up of the following segments of DNA. The first is a 0.93 kb *Ava*I to engineered-*Eco*RV fragment isolated from transposon Tn7 that encodes bacterial spectinomycin/streptomycin resistance (Spc/Str), which is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*. This is joined to the 1.61 kb segment of DNA encoding a chimeric kanamycin resistance which permits selection of transformed plant cells. The chimeric gene (P-35S/KAN/NOS 3') consists of the cauliflower mosaic virus (CaMV) 35S promoter, the neomycin phosphotransferase typeII (KAN) gene, and the 3'-nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983). The next segment is the 0.75 kb *ori*V containing the origin of replication from the RK2 plasmid. It is joined to the 3.1 kb *Sal*I to *Pvu*I segment of pBR322 (*ori*322) which provides the origin of replication for maintenance in *E. coli* and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells. The next segment is the 0.36 kb *Pvu*I to *Bcl*I from pTiT37 that carries the nopaline-type T-DNA right border (Fraley et al., 1985).

The pMON981 plasmid contains the following DNA segments: the 0.93 kb fragment isolated from transposon Tn*7* encoding bacterial spectinomycin/streptomycin resistance [Spc/Str; a determinant for selection in *E. coli* and *Agrobacterium tumefaciens* (Fling et al., 1985)]; the chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue, consisting of the 0.35 kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 kb neomycin phosphotransferase typeII gene (KAN), and the 0.26 kb 3'-nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983); the 0.75 kb origin of replication from th RK2 plasmid (*ori*V) (Stalker et al., 1981); the 3.1 kb *Sal*I to *Pvu*I segment of pBR322 which provides the

origin of replication for maintenance in *E. coli* (*ori*-322) and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells, and the 0.36 kb *Pvu*I to *Bcl*I fragment from the pTiT37 plasmid containing the nopaline-type T-DNA right border region (Fraley et al., 1985). The expression cassette consists of the 0.6 kb 35S promoter from the figwort mosaic virus (P-FMV) (Gowda et al., 1989) and the 0.7 kb 3' non-translated region of the pea rbcS-E9 gene (E9 3') (Coruzzi et al., 1984, and Morelli et al., 1985). The 0.6 kb *Ssp*I fragment containing the FMV35S promoter (Figure 1) was engineered to place suitable cloning sites downstream of the transcriptional start site.

The plant vector was mobilized into the ABI *Agrobacterium* strain. The ABI strain is the A208 *Agrobacterium tumefaciens* carrying the disarmed Ti plasmid pTiC58 (pMP90RK) (Koncz and Schell, 1986). The Ti plasmid does not carry the T-DNA phytohormone genes and the strain is therefore unable to cause the crown gall disease. Mating of the plant vector into ABI was done by the triparental conjugation system using the helper plasmid pRK2013 (Ditta et al., 1980). When the plant tissue is incubated with the ABI::plant vector conjugate, the vector is transferred to the plant cells by the *vir* functions encoded by the disarmed pTiC58 plasmid. The vector opens at the T-DNA right border region, and the entire plant vector sequence may be inserted into the host plant chromosome. The pTiC58 Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium*.

PLANT REGENERATION

When adequate production of the glyphosate oxidoreductase activity is achieved in transformed cells (or protoplasts), the cells (or protoplasts) are regenerated into

whole plants. Choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from Leguminosae (alfalfa, soybean, clover, etc.), Umbelliferae (carrot, celery, parsnip), Cruciferae (cabbage, radish, rapeseed, etc.), Cucurbitaceae (melons and cucumber), Gramineae (wheat, rice, corn, etc.), Solanaceae (potato, tobacco, tomato, peppers) and various floral crops. See, e.g., Ammirato, 1984; Shimamoto, 1989; Fromm, 1990; Vasil, 1990.

The following examples are provided to better elucidate the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications, truncations, etc. can be made to the methods and genes described herein while not departing from the spirit and scope of the present invention.

EXAMPLES

Expression, Activity and Phenotype of Glyphosate Oxidoreductase in Transformed Plants

The transformation, expression and activity of glyphosate oxidoreductase, and the glyphosate tolerance phenotype imparted to the plants by the glyphosate oxidoreductase genes, introduced into *Nicotiana tabacum* cv. "Samsun" and/or *Brassica napus* cv. Westar using the vectors pMON17073, pMON17032, pMON17065, pMON17066, pMON17138, and pMON17164, is described in the following exemplary embodiments. Initial data in tobacco on the expression of the manipulated glyphosate oxidoreductase gene (SEQ ID NO:6) under the control of the En-CaMV35S promoter (see data on pMON17073 in Tables VIII and IX, for example) indicated only l w levels of expression of glyphosate oxidoreductase. The transcription of the gene was confirmed in

the case of 3-4 plants by Northern and S1 analysis but no glyphosate oxidoreductase protein could be detected (limit of detection in that assay was ~0.01% expression level). Analysis of R_0 plants following spray with 0.4 lb/acre (approximately 0.448 kg/ha) glyphosate also showed only low levels of tolerance. Modification of the gene sequence (as described herein) resulted in improved expression in tobacco, as did the use of the FMV promoter and the use of a CTP fusion to the glyphosate oxidoreductase gene. For these reasons the majority of the data presented comes from transgenic plants derived using vectors containing these improved glyphosate oxidoreductase constructs. One set of experiments with the modified glyphosate oxidoreductase vector pMON17032 are presented in example 1 and a study of manipulated glyphosate oxidoreductase, synthetic glyphosate oxidoreductase, and CTP1-synthetic glyphosate oxidoreductase is presented in example 2. The transformation and expression of glyphosate oxidoreductase in canola is described in example 3.

Example 1

The tobacco leaf disc transformation protocol employs healthy leaf tissue about 1 month old. After a 15-20 minute surface sterilization with 10% Clorox plus a surfactant, the leaves were rinsed 3 times in sterile water. Using a sterile paper punch, leaf discs are punched and placed upside down on MS104 media (MS salts 4.3 g/l, sucrose 30 g/l, B5 vitamins 500X 2 ml/l, NAA 0.1 mg/l, and BA 1.0 mg/l) for a 1 day preculture.

The discs were then inoculated with an overnight culture of disarmed *Agrobacterium* ABI containing the subject vector that had been diluted 1/5 (ie: about 0.6 OD). The inoculation was done by placing the discs in centrifuge tubes with the culture. After 30 to 60 seconds, the liquid was drained

off and the discs were blotted between sterile filter paper. The discs were then placed upside down on MS104 feeder plates with a filter disc to co-culture.

After 2-3 days of co-culture, the discs were transferred, still upside down, to selection plates with MS104 media. After 2-3 weeks, callus formed, and individual clumps were separated from the leaf discs. Shoots were cleanly cut from the callus when they were large enough to distinguish from stems. The shoots were placed on hormone-free rooting media (MSO: MS salts 4.3 g/l, sucrose 30 g/l, and B5 vitamins 500X 2 ml/l) with selection. Roots formed in 1-2 weeks. Any leaf callus assays are preferably done on rooted shoots while still sterile. Rooted shoots were placed in soil and were kept in a high humidity environment (ie: plastic containers or bags). The shoots were hardened off by gradually exposing them to ambient humidity conditions.

A total of 45 Kanamycin resistant pMON17032 tobacco lines were examined (Table V).

TABLE V - Expression of Modified Glyphosate Oxidoreductase Gene in Tobacco

(R1 Transgenics of pMON17032)

# Plants	Glyphosate Recallusing (0.5mM glyphosate)			Western Analysis of Plants‡	
	+	+/-	-	+	-
45	0	11	34	24	21

‡+ means 0.5 - 2 ng/50 μg protein
– means <0.5 ng/50 μg protein

Leaf recallusing on plant tissue culture media indicated a low level of glyphosate tolerance (rated as a +/- phenotype) for at least 11 of these lines. At least 24 of these lines expressed a detectable level of glyphosate oxidoreductase in the range of 0.5 to 2 ng per 50 μg of extractable protein. The glyphosate tolerance displayed in the leaf recallusing assay and the higher glyphosate oxidoreductase expression level indicate that the changes made to the glyphosate oxidoreductase coding sequences to make the modified glyphosate oxidoreductase gene (SEQ ID NO:7) had a marked effect on the ability of this gene to be expressed in plants. This same effect could also then be achieved by expressing the manipulated glyphosate oxidoreductase gene (SEQ ID NO:6) using stronger plant promoters, using better 3' polyadenylation signal sequences, optimizing the sequences around the initiation codon for ribosome loading and translation initiation, or by combinations of these or other expression or regulatory sequences or factors. The R1 progeny of a number of these lines, including those with the highest glyphosate oxidoreductase expression level (#'s 18854 and 18848) were sprayed with glyphosate at rates of 0.4 and 1.0 lb/acre (0.448 and 1.12 kg/ha, respectively) and vegetative performance rated over a period of four weeks (Table VI).

TABLE VI - Tobacco Spray Data for pMON17032 R1 Plants

Line	Rate kg/ha	Vegetative Score * 7 Days	14 Days	28 Days
18860	0.448	3	3	4
	1.12	1	1	2
18842	0.448	4	6	8
	1.12	2	3	6
18848	0.448	3	4	8
	1.12	2	2	6
18854	0.448	4	7	9
	1.12	2	5	8
18858	0.448	3	4	6
	1.12	1	2	4
18885	0.448	4	5	8
	1.12	2	1	2
18890	0.448	3	6	7
	1.12	1	2	3
Samsun	0.448	1	1	2
	1.12	1	1	0

* Vegetative Score

0 = Dead

10 = No detectable effect

Following an initial lag, and especially for those plants expressing the highest levels of glyphosate oxidoreductase, these lines showed vegetative glyphosate tolerance at both spray rates (that improved with time). Glyphosate oxidoreductase enzyme activity was determined for two of the pMON17032 lines (#'s 18858 and 18881). Leaf tissue (1g) was harvested, frozen in liquid N_2, and stored at -80°C prior to extracti n. For extraction, leaf tissue was pulv rized in a

mortar and pestle with liquid N_2. To the powdered leaf tissue was then added 1 ml extraction buffer (100 mM TrisCl, pH 7.4, 1 mM EDTA, 20% glycerol, 35 mM KCl, 1 mM benzamidine HCl, 5 mM Na ascorbate, 5 mM dithiothreitol, and 1 mg/ml bovine serum albumin, 4°C), and the sample was further ground for 1 minute. The resulting mixture was centrifuged for 5 minutes (high speed, Eppendorf) and the supernatant was treated with a saturated ammonium sulfate solution to give 70% final saturation (2.33 ml saturated solution/ml extract). The precipitated protein was collected by centrifugation as above, and the pellet was resuspended in 0.4 ml of extraction buffer. After centrifuging again to remove particulate matter, the sample was desalted using Sephadex G50 contained in a 1 ml syringe, equilibrated with extraction buffer, according to the method of Penefsky (1979). The desalted plant extracts were stored on ice, and protein concentrations were determined by the method of Bradford (1976). Glyphosate oxidoreductase reactions were carried out in duplicate for 60 minutes at 30°C in an assay mixture of 0.1 MOPS/0.01 tricine buffer, pH 7.4, containing 10 mM $MgCl_2$, 0.01 mM flavin adenine dinucleotide (FAD, Sigma), and 1 mM ubiquinone Qo, (Sigma). Plant extracts (75 µl) were preincubated in the assay mixture for 2 minutes, and reactions were then initiated by adding iminodiacetic acid (IDA, 20 µl) substrate to a final concentration of 50 mM (total assay volume was 0.2 ml). Reactions were quenched and derivatized as described below. Control reactions omitting IDA and omitting plant extract were also performed. Glyoxylate detection was carried out using 2,4-dinitrophenylhydrazine (2,4-DNPH) derivatization and reverse phase high performance liquid chromotography (HPLC), using a modification of the method of Qureshi et al. (1982). Glyphosate oxidoreductase reactions (0.2 ml) were qu nched

with 0.25 ml of DNPH reagent (0.5 mg/ml DNPH [Aldrich] in 0.5 M HCl) and allowed to derivatize for 5 minutes at 25°C. The samples were then extracted with ethyl acetate (2 x 0.3ml) and the combined ethyl acetate extracts were extracted with 10% Na_2CO_3 (0.3 ml). The Na_2CO_3 phase was then washed once with ethyl acetate (0.2 ml) and the Na_2CO_3 phase injected (100 µl) on a Beckman Ultrasphere C18 IP HPLC column (5 µ, 4.6 mm x 25 cm) using an LKB GTi binary HPLC system with a Waters 990 photodiode array UV/VIS HPLC detector, via a Waters WISP HPLC autoinjector. The isocratic mobile phase was methanol-water-acetic acid (60:38.5:1.5) with 5 mM tetrabutylammonium phosphate (Pierce). The DNPH-glyoxylate peak (retention time = 6.7 minutes) was detected at 365 nm and compared to a glyoxylate standard (Sigma, 20 µM in 0.2 ml) derivatized in exactly the same manner.

<u>TABLE VII - Glyphosate oxidoreductase Activity of Transgenic Tobacco Plants</u>

Plant	Specific Activity nmol/min mg
Samsun	0 (not detectable)
18881	0.039
18858	0.018

<u>Example 2</u>

A series of transformed tobacco lines were derived using the "isogenic" glyphosate oxidoreductase vectors pMON17073 (manipulated glyphosate oxidoreductase) (SEQ ID NO:6), pMON17065 (synthetic glyphosate oxidoreductas) (SEQ ID NO:8), and pMON17066 (CTP1-synthetic glyphosate

oxidoreductase). By Western analysis (see Table VII below) of a number of these lines, the manipulated glyphosate oxidoreductase plants were found to express up to ~0.5 ng glyphosate oxidoreductase per 50 μg plant protein, the synthetic glyphosate oxidoreductase at levels from ~0.5 - 2 ng per 50 μg, and at levels from ~2 - 20 ng per 50 μg for the CTP1-synthetic glyphosate oxidoreductase plants.

TABLE VIII - Glyphosate Oxidoreductase Expression in Tobacco

Construct	Plant#	Western Rating
pMON17073 (manipulated)	21270	0
	21281	0
	21286	1
	21929	1
pMON17066 (CTP1-synthetic)	21237	1
	21830	0
	21845	3
	21872	3
	21889	1
	21891	0
pMON17065 (synthetic)	21199	0
	21208	2
	21211	2
	21217	0
	21218	2
	21792	1
	21795	0
	21811	2

Western rating scale per 50 µg of protein:
O - no detectable glyphosate oxidoreductase
1 - <.5ng
2 - .5ng - 2ng
3 - >2ng

A number of primary transformants R_0 lines, expressing manipulated or synthetic glyphosate oxidoreductase or CTP1-synthetic glyphosate oxidoreductase, were sprayed with glyphosate at 0.4 lb/acre (0.448 kg/ha) and rated as before.

TABLE IX - Glyphosate Spray Data: pMON17066
(CTP1-Glyphosate Oxidoreductase)
Tobacco (R_0 plants)

Line	Western Rating	Vegetative Score# (Spray Rate = 0.4 lb/acre) (0.448 kg/ha) 7	14	28 (days after spray)	
Control A	0	3	0	0	no detectable
Control B	0	3	1	0	glyphosate
Control C	0	3	1	1	oxidoreductase
22933	1	3	1	0	(pMON17073)
22741	2	2	1	9	(pMON17065)
22810	3	3	4	6	(pMON17066)
22825	1	2	1	1	(pMON17066)
22822	3	10	10	10	(pMON17066)
22844	3	10	10	10	(pMON17066)
22854	3	9	10	10	(pMON17066)
22860	3	8	10	10	(pMON17066)
22880	1	3	2	9	(pMON17066)
22881	2	2	0	0	(pMON17066)
22886	3	9	10	10	(pMON17066)
22887	3	9	10	10	(pMON17066)

Western rating scale
(per 50 µg protein)
0 = no detectable glyphosate oxidoreductase
1 = <0.5ng
2 = 0.5 - 2ng
3 = >2ng

# Vegetative score: 0 = dead;
10 = no detectable effect

The synthetic glyphosate oxidoreductase line displayed a response similar to that noted for the modified glyphosate oxidoreductase R_1 plants, in that there was some immediate glyphosate effects that were overcome with time, through the metabolism of the herbicide by glyphosate oxidoreductase to the derivatives AMPA and glyoxylate. Since the target of glyphosate (EPSP synthase) is located in the chloroplast, the activity of glyphosate oxidoreductase must be reducing the level of glyphosate within this organelle by removing the herbicide before it reaches the chloroplast. The CTP1-synthetic glyphosate oxidoreductase plants displayed a superior glyphosate tolerance in that these plants did not show much, if any, immediate glyphosate effects at the treated rate. In general, the treated tolerant plants also showed normal development, flowering and fertility.

The CTP1-synthetic glyphosate oxidoreductase plants showed a markedly higher level of glyphosate oxidoreductase expression than that shown for the other glyphosate oxidoreductase constructs. This increased glyphosate oxidoreductase level could be due to enhancement of translation of the fusion or to sequestering of glyphosate oxidoreductase within the chloroplast and leading to a longer protein half-life. The higher level of glyphosate oxidoreductase and/or its location in the chloroplast can result in higher levels of glyphosate tolerance through rapid detoxification of glyphosate in the chloroplast. The presence of glyphosate oxidoreductase within the chloroplast has been confirmed. Five leaves from each of four plants (#22844, 22854, 22886, 22887), shown to be Western positive for glyphosate oxidoreductase, were homogenized in Waring blender in 0.9 L GR+ buffer (Bartlett, et al., 1982) for 3 X 3 seconds at high speed. The homogenate was filtered through 4 layers of Miracloth and centrifuged at 6,000

rpm in a GS-3 rotor. The pellet was resuspended in 4 ml total of GR+ buffer and placed on top of a 40/80% Percoll step gradient and spun at 9,500 rpm for 10 minutes. The intact chloroplasts (lower band) were washed once with GR- buffer (Bartlett, et al., 1982) and centrifuged (up to 6,000 rpm with brake off). They were then resuspended in 300 µl 50 mM Hepes pH 7.7, 330 mM Sorbitol and lysed on ice using by sonication (small probe, 30%-3 microtip setting x 10 seconds). The debris was pelleted and the supernatant passed through a Sephadex G50 column into 50 mM Hepes, pH 7.5. The soluble protein concentration was 2.4 mg/ml. The enzyme assays were done as above using both 50 mM IDA and 50 mM glyphosate as substrates (30 minute assays), but without the addition of 1 mM ubiquinone.

Table IX - Glyphosate Oxidoreductase Activity in Isolated Chloroplast from Transgenic Tobacco

Substrate	Specific Activity (nmoles/min.mg)
Iminodiacetic acid	179
Glyphosate	92

Example 3

A number of transformed lines of canola have been derived with vectors pMON17138 (CTP1-synthetic glyphosate oxidoreductase) and pMON17164 (CTP2-synthetic glyphosate oxidoreductase) as follows.

Plant Material

Seedlings of *Brassica napus* cv *Westar* were established in 2 inch (~ 5 cm) pots containing Metro Mix 350.

They were grown in a growth chamber at 24°C, 16/8 hour photoperiod, light intensity of 400 uEm^{-2}sec^{-1} (HID lamps). They were fertilized with Peters 20-10-20 General Purpose Special. After 2 1/2 weeks they were transplanted to 6 inch (~ 15 cm) pots and grown in a growth chamber at 15/10°C day/night temperature, 16/8 hour photoperiod, light intensity of 800 uEm^{-2}sec^{-1} (HID lamps). They were fertilized with Peters 15-30-15 Hi-Phos Special.

Transformation/Selection/Regeneration

Four terminal internodes from plants just prior to bolting or in the process of bolting but before flowering were removed and surfaced sterilized in 70% v/v ethanol for 1 minute, 2% w/v sodium hypochlorite for 20 minutes and rinsed 3 times with sterile deionized water. Stems with leaves attached could be refrigerated in moist plastic bags for up to 72 hours prior to sterilization. Six to seven stem segments were cut into 5mm discs with a Redco Vegetable Slicer 200 maintaining orientation of basal end.

The *Agrobacterium* was grown overnight on a rotator at 24°C in 2mls of Luria Broth containing 50mg/l kanamycin, 24mg/l chloramphenicol and 100mg/l spectinomycin. A 1:10 dilution was made in MS (Murashige and Skoog) media giving approximately 9x10^8 cells per ml. This was confirmed with optical density readings at 660 mu. The stem discs (explants) were inoculated with 1.0ml of *Agrobacterium* and the excess was aspirated from the explants.

The explants were placed basal side down in petri plates containing 1/10X standard MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1.0mg/l 6-benzyladenine (BA). The plates were layered with 1.5ml of m dia containing MS salts, B5

vitamins, 3% sucrose, pH 5.7, 4.0mg/l p-chlorophenoxyacetic acid, 0.005mg/l kinetin and covered with sterile filter paper.

Following a 2 to 3 day co-culture, the explants were transferred to deep dish petri plates containing MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1mg/l BA, 500mg/l carbenicillin, 50mg/l cefotaxime, 200 mg/l kanamycin or 175mg/l gentamicin for selection. Seven explants were placed on each plate. After 3 weeks they were transferred to fresh media, 5 explants per plate. The explants were cultured in a growth room at 25°C, continuous light (Cool White).

Expression Assay

After 3 weeks shoots were excised from the explants. Leaf recallusing assays were initiated to confirm modification of R_o shoots. Three tiny pieces of leaf tissue were placed on recallusing media containing MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 5.0mg/l BA, 0.5mg/l naphthalene acetic acid (NAA), 500mg/l carbenicillin, 50mg/l cefotaxime and 200mg/l kanamycin or gentamicin or 0.5mM glyphosate. The leaf assays were incubated in a growth room under the same conditions as explant culture. After 3 weeks the leaf recallusing assays were scored for herbicide tolerance (callus or green leaf tissue) or sensitivity (bleaching).

Transplantation

At the time of excision, the shoot stems were dipped in Rootone® and placed in 2 inch (~ 5 cm) pots containing Metro-Mix 350 and placed in a closed humid environment. They were placed in a growth chamber at 24°C, 16/8 hour photoperiod, 400 $uEm^{-1}sec^{-2}$(HID lamps) for a hardening-off period of approximately 3 weeks.

The seed harvested from R_o plants is R_1 seed which gives rise to R_1 plants. To evaluate the glyphosate tolerance of an R_o plant, its progeny are evaluated. Because an R_o plant is assumed to be hemizygous at each insert location, selfing results in maximum genotypic segregation in the R_1. Because each insert acts as a dominant allele, in the absence of linkage and assuming only one hemizygous insert is required for tolerance expression, one insert would segregate 3:1, two inserts, 15:1, three inserts 63:1, etc. Therefore, relatively few R_1 plants need be grown to find at least one resistant phenotype.

Seed from an R_o plant is harvested, threshed, and dried before planting in a glyphosate spray test. Various techniques have been used to grow the plants for R_1 spray evaluations. Tests are conducted in both greenhouses and growth chambers. Two planting systems are used; ~ 10 cm pots or plant trays containing 32 or 36 cells. Soil used for planting is either Metro 350 plus three types of slow release fertilizer or plant Metro 350. Irrigation is either overhead in greenhouses or subirrigation in growth chambers. Fertilizer is applied as required in irrigation water. Temperature regimes appropriate for canola were maintained. A sixteen hour photoperiod was maintained. At the onset of flowering, plants are transplanted to ~15 cm pots for seed production.

A spray "batch" consists of several sets of R_1 progenies all sprayed on the same date. Some batches may also include evaluations of other than R_1 plants. Each batch also includes sprayed and unsprayed non-transgenic genotypes representing the genotypes in the particular batch which were putatively transformed. Also included in a batch is one or more non-segregating transformed genotypes previously identified as having some resistance.

Two-six plants from each individual R_o progeny are not sprayed and serve as controls to compare and measure the glyphosate tolerance, as well as to assess any variability not induced by the glyphosate. When the other plants reach the 2-4 leaf stage, usually 10 to 20 days after planting, glyphosate is applied at rates varying from 0.28 to 1.12 kg/ha, depending on objectives of the study. Low rate technology using low volumes has been adopted. A laboratory track sprayer has been calibrated to deliver a rate equivalent to field conditions.

A scale of 0 to 10 is used to rate the sprayed plants for vegetative resistance. The scale is relative to the unsprayed plants from the same R_o plant. A 0 is death, while a 10 represents no visible difference from the unsprayed plant. A higher number between 0 and 10 represents progressively less damage as compared to the unsprayed plant. Plants are scored at 7, 14, and 28 days after treatment (DAT), or until bolting, and a line is given the average score of the sprayed plants within an R_o plant family.

Six integers are used to qualitatively describe the degree of reproductive damage from glyphosate:

0: No floral bud development

2: Floral buds present, but aborted prior to opening

4: Flowers open, but no anthers, or anthers fail to extrude past petals

6: Sterile anthers

8: Partially sterile anthers

10: Fully fertile flowers

Plants are scored using this scale at or shortly after initiation of flowering, depending on th rate of floral structure development.

Tables X and XI below tabulate the vegetative and reproductive scores for canola plants transformed with pMON17138 (sprayed at a rate of 0.56 kg/ha and pMON17164 (sprayed at a rate of 0.84 kg/ha), respectively. The results presented below illustrate the glyphosate tolerance conferred to canola plants as a result of expression of a glyphostate oxidreductase gene in the plants.

Table X - Glyphosate Spray Evaluation of Canola Plants containing pMON17138

Line name	Batch	0.56 kg/ha score 14 DAT Vegetative	0.56kg/ha score 28 DAT Reproductive
17138-22	79	9	10
17138-30	79	9	10
17138-145	79	10	10
17138-158	79	8	10
17138-164	80	8	10
Untransformed	77	3	0
Untransformed	79	1	0

Table XI - Glyphosate Spray Evaluation of Canola Plants containing pMON17164

Construct	Batch	0.84 kg/ha score 14 DAT vegetative	28 DAT reproductive
17164-6	82	7	10
17164-9	83	8	10
17164-20	82	7	10
17164-25	83	8	10
17164-35	84	7	10
17164-45	83	9	10
17164-61	83	7	10
17164-75	84	7	10
17164-85	84	7	10
17164-97	84	6	10
17164-98	83	9	10
17164-105	83	7	10
17164-110	83	9	10
17164-115	83	7	10
17164-129	83	8	10
17164-139	84	7	10
17164-140	83	8	10
17164-164	83	7	10
17164-166	83	8	10
17164-174	83	8	10
17164-186	83	3	10
17164-202	83	8	10
17164-218	84	6	10
17164-219	83	9	10
17164-222	84	7	10

17164-225	83	7	10
17164-227	84	7	10
17164-230	83	8	10
17164-243	83	7	10
17164-247	84	7	10
17164-287	84	7	10
17164-289	83	8	10
17164-300	83	9	10
17164-337	83	8	10

Example 4

The glyphosate oxidoreductase gene has also been introduced into and expressed in soybean and imparts glyphosate tolerance to such plants. The CTP2-synthetic glyphosate oxidoreductase fusion gene (as described above) was introduced into soybean under the control of the FMV promoter and with the NOS 3' sequences in vector pMON17159, a map of which is presented in Figure 10. This vector consists of the following elements in addition to the glyphosate oxidoreductase gene sequences; the pUC origin of replication, an NPTII bacterial selectable marker gene (kanamycin) and the beta-glucuronidase gene (GUS; Jefferson et a. 1986) under the control of the E35S promoter and with the E9 3' sequences. The latter gene provides a scorable marker to facilitate the identification of transformed plant material.

Soybean plants are transformed with pMON17159 by the method of microprojectile injection using particle gun technology as described in Christou et al. (1988). The seed harvested from R_0 plants is R_1 seed which gives rise to R_1 plants. To evaluate the glyphosate tolerance of an R_0 plant, its progeny are evaluated. Because an R_0 plant is assumed to be hemizygous at each insert location, selfing r sults in

maximum genotypic segregation in the R_1. Because each insert acts as a dominant allele, in the absence of linkage and assuming only one hemizygous insert is required for tolerance expression, one insert would segregate 3:1, two inserts, 15:1, three inserts 63:1, etc. Therefore, relatively few R_1 plants need be grown to find at least one resistant phenotype.

Seed from an R_o soybean plant is harvested, and dried before planting in a glyphosate spray test. Seeds are planted into 4 inch (~5cm) square pots containing Metro 350. Twenty seedlings from each Ro plant is considered adequate for testing. Plants are maintained and grown in a greenhouse environment. A 12.5-14 hour photoperiod and temperatures of 30°C day and 24°C night is regulated. Water soluble Peters Pete Lite fertilizer is applied as needed.

A spray "batch" consists of several sets of R_1 progenies all sprayed on the same date. Some batches may also include evaluations of other than R_1 plants. Each batch also includes sprayed and unsprayed non-transgenic genotypes representing the genotypes in the particular batch which were putatively transformed. Also included in a batch is one or more non-segregating transformed genotypes previously identified as having some resistance.

One to two plants from each individual R_o progeny are not sprayed and serve as controls to compare and measure the glyphosate tolerance, as well as to assess any variability not induced by the glyphosate. When the other plants reach the first trifoliolate leaf stage, usually 2-3 weeks after planting, glyphosate is applied at a rate equivalent of 128 oz./acre (8.895kg/ha) of Roundup®. A laboratory track sprayer has been calibrated to deliver a rate equivalent to those conditions.

A vegetative score of 0 to 10 is used. The score is relative to the unsprayed progenies from the same R plant. A

0 is death, while a 10 represents no visible difference from the unsprayed plant. A higher number between 0 and 10 represents progressively less damage as compared to the unsprayed plant. Plants are scored at 7, 14, and 28 days after treatment (DAT).

Table XII - Glyphosate Spray Evaluation of Soybean Plants containing pMON17159

Line	Batch	Score @ 8.895kg/ha, 28 DAT
17159-24	14	9
17159-25	14	9
17159-28	14	6
17159-40	14	4
17159-43	14	4
17159-71	14	10
17159-77	14	9
17159-81	15	4
Untransformed	14	0

Example 5

The glyphosate oxidoreductase gene has also been introduced into Black Mexican Sweet (BMS) corn cells with expression of the protein detected in callus.

Plasmid pMON19632 was used to introduce the glyphosate oxidoreductase gene into corn cells. The backbone for this plasmid was constructed by inserting the 0.6kb cauliflower mosaic virus (CaMV) 35S RNA promoter (E35S) containing a duplication of the -90 to -300 region (Kay et al., 1987), a 0.58kb fragment containing the first intron from the maize alcohol dehydrogenase gene (Callis et al., 1987), and the 3' terminati n sequences from the nopaline synthase (NOS) gene (Fraley et al., 1983) into pUC119 (Yanisch-Perron et al.,

1985). pMON19632 was formed by inserting the 1.7kb BglII/EcoRI fragment from pMON17064 which contains the *Arabidopsis* SSU CTP fused to the synthetic glyphosate oxidoreductase coding sequence (SEQ IN NO:8).

Plasmid pMON19632 was introduced into BMS corn cells by co-bombardment with EC9, a plasmid containing a sulfonylurea-resistant form of the maize acetolactate synthase gene. 2.5 μg of each plasmid was coated onto tungsten particles and introduced into log-phase BMS cells using a PDS-1000 particle gun essentially as described in Klein et al., 1989. Transformants were selected on MS medium containing 20ppb chlorsulfuron. After initial selection on chlorsulfuron, the calli was assayed by glyphosate oxidoreductase Western blot.

BMS callus (3 g wet weight) was dried on filter paper (Whatman#1) under vacuum, reweighed, and extraction buffer (500 μl/g dry weight; 100 mM Tris, 1 mM EDTA, 10% glycerol) was added. The tissue was homogenized with a Wheaton overhead stirrer for 30 seconds at 2.8 power setting. After centrifugation (3 minutes, Eppendorf microfuge), the supernatant was removed and the protein was quantitated (BioRad Protein Assay). Samples (50 μg/well) were loaded on an SDS PAGE gel (Jule, 3-17%) along with glyphosate oxidoreductase standard (10 ng), electrophoresed, and transferred to nitrocellulose similarly to a previously described method (Padgette, 1987). The nitrocellulose blot was probed with goat anti-glyphosate oxidoreductase IgG, and developed with I-125 Protein G. The radioactive blot was visualized by autoradiography. Results were quantitated by densitometry on an LKB UltraScan XL laser densitomer and are tabulated below in Table XIII.

Table XIII - Expression of glyphosate oxidoreductase in BMS Corn Callus using pMON19632

Line	GOX expression (% extracted protein)
EC9 (no GOX)	0
T13-17	0.016
T13-16	0.0065
T13-15	0.016
T13-14	0.003
T13-12	0.0079
T13-7	0.01
T13-5	0.004
T13-18	0.026
T13-8	0.019
T13-9	0.01
T13-4	0.027

Table XIII illustrates that glyphosate oxidoreductase can be expressed and detected in a monocotyledonous plant, such as corn.

Example 6

The glyphosate oxidoreductase gene may be used as a selectable marker for plant transformation directly on media containing glyphosate. The ability to select and to identify transformed plant material depends, in most cases, on the use of a dominant selectable marker gene to enable the preferential and continued growth of the transformed tissues in the presence of a normally inhibitory substance. Antibiotic resistance and herbicide tolerance genes have been used almost exclusively as such dominant selectable marker genes in the presence of the corresponding antibiotic or herbicide. Th

nptII/kanamycin selection scheme is probably the most frequently used. It has been demonstrated that glyphosate oxidoreductase is also a useful and perhaps superior selectable marker/selection scheme for producing and identifying transformed plants.

A plant transformation vector that may be used in this scheme is pMON17226 (Figure 11). This plasmid resembles many of the other plasmids described infra and is essentially composed of the previously described bacterial replicon system that enables this plasmid to replicate in *E. coli* and to be introduced into and to replicate in *Agrobacterium*, the bacterial selectable marker gene (Spc/Str), and located between the T-DNA right border and left border is the CTP1-glyphosate oxidoreductase synthetic gene in the FMV promoter-E9 3' cassette. This plasmid also has single sites for a number of restriction enzymes, located within the borders and outside of the expression cassette. This makes it possible to easily add other genes and genetic elements to the vector for introduction into plants.

The protocol for direct selection of transformed plants on glyphosate is outlined for tobacco. Explants are prepared for pre-culture as in the standard procedure as described in Example 1: surface sterilization of leaves from 1 month old tobacco plants (15 minutes in 10% clorox + surfactant; 3X dH_2O washes); explants are cut in 0.5 x 0.5 cm squares, removing leaf edges, mid-rib, tip, and petiole end for uniform tissue type; explants are placed in single layer, upside down, on MS104 plates + 2 ml 4COO5K media to moisten surface; pre-culture 1-2 days. Explants are inoculated using overnight culture of *Agrobacterium* containing the plant transformation plasmid that is adjusted to a titer of 1.2×10^9 bacteria/ml with 4COO5K media. Explants are placed into a centrifuge tube, the

Agrobacterium suspension is added and the mixture of bacteria and explants is "Vortexed" on maximum settting for 25 seconds to ensure even penetration of bacteria. The bacteria are poured off and the explants are blotted between layers of dry sterile filter paper to remove excess bacteria. The blotted explants are placed upside down on MS104 plates + 2ml 4COO5K media + filter disc. Co-culture is 2-3 days. The explants are transferred to MS104 + Carbenicillin 1000 mg/l + cefotaxime 100 mg/l for 3 days (delayed phase). The explants are then transferred to MS104 + glyphosate 0.05 mM + Carbenicillin 1000 mg/l + cefotaxime 100 mg/l for selection phase. At 4-6 weeks shoots are cut from callus and placed on MSO + Carbenicillin 500 mg/l rooting media. Roots form in 3-5 days, at which time leaf pieces can be taken from rooted plates to confirm glyphosate tolerance and that the material is transformed.

The presence of the glyphosate oxidoreductase protein in these transformed tissues has been confirmed by immunoblot analysis of leaf discs. The data from one experiment with pMON17226 is presented in the following: 25 shoots formed on glyphosate from 100 explants inoculated with *Agrobacterium* ABI/pMON17226; 15 of these were positive on recallusing on glyphosate, and 19 of these were positive for glyphosate oxidoreductase protein as detected by immunoblot. These data indicate a transformation rate of 15-19 per 100 explants, which makes this a highly efficient and time saving transformation procedure for plant. Similar transformation frequencies have been obtained with a pMON17226 derivative (pMON17241) containing the gene for the glyphosate oxidoreductase v.247 (SEQ ID NO:17). The glyphosate oxidoreductase gene has also been shown to enable direct selection of transformants in other plant sp cies, including *Arabidopsis*, potato, and sugarbeet.

From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with advantages which are obvious and which are inherent to the invention.

It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.

Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

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SEQUENCE LISTING

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Barry, Gerard F.

(ii) TITLE OF INVENTION: Glyphosate Resistant Plants

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release Ø1.0, Version Ø1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 564 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATTTAGCAGC ATTCCAGATT GGGTTCAATC AACAAGGTAC GAGCCATATC ACTTTATTCA 60

AATTGGTATC GCCAAAACCA AGAAGGAACT CCCATCCTCA AAGGTTTGTA AGGAAGAATT 120

CTCAGTCCAA AGCCTCAACA AGGTCAGGGT ACAGAGTCTC CAAACCATTA GCCAAAAGCT 180

ACAGGAGATC AATGAAGAAT CTTCAATCAA AGTAAACTAC TGTTCCAGCA CATGCATCAT 240

GGTCAGTAAG TTTCAGAAAA AGACATCCAC CGAAGACTTA AAGTTAGTGG GCATCTTTGA 300

AAGTAATCTT GTCAACATCG AGCAGCTGGC TTGTGGGGAC CAGACAAAAA AGGAATGGTG 360

CAGAATTGTT AGGCGCACCT ACCAAAAGCA TCTTTGCCTT TATTGCAAAA GATAAAGCAG 420

ATTCCTCTAG TACAAGTGGG GAACAAAATA ACGTGGAAAA GAGCTGTCCT GACAGCCCAC 480

TCACTAATGC GTATGACGAA CGCAGTGACG ACCACAAAAG AATTTTCCCT CTATATAAGA 540

AGGCATTTCA TTCCCATTTG AAGG 564

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCATCAGAT ACTAACCAAT ATTTCTC 27

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1689 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NCATGGACGT CTGATCGAAA TCGTCGTTAC CGCAGCAAGG TAAGGCACGC CGAATTTTAT 60
CACCTACCGC GAAACGGTGG CTAGGCAGCG AGAGACTGTC GGCTCCGCGG GAGCATCCTA 120
TGTCTGAGAA CCACAAAAAA GTAGGCATCG CTGGAGCCGG AATCGTCGGC GTATGCACGG 180

CGCTGATGCT TCAGCGCCGC CGATTCAAAG TCACGTTGAT TGACCCGAAC GCTGCTGGCG 240

AACGTGCATC GTTTGCGAAT GCGCGATGCT TCAACGGCTC ATCGGTCGTC GCTATGTCCA 300
TGCCCGGAAA CTTGACGAGC GTGCCGAAGT CGCTGCTTGA CCCGATGCGC CGTTGTCAAT 360

CCCGTTCAGC TATTTCCAAC CATCATGCCT GGTTGATTCG CTTTCTGTTA GCCGGAAGAC 420

CAAACAAGGT GAAGGAGCAG GCGAAAGCAC TGCGCAATGT CATCAAGTCC ACGGTGCCTC 480

TGATCAAGTC ATTCGCCGAG GACGCTGATG CGAGCCATCT GATCGGCCAT GAAGGTCATC 540

TGACCGTATA TCGTGGAGAA GCAGACTTCG GCAAGGACGG CCGACGTTGG GAACTGCCGC 600

GTCTCAACGG TGTTCGCACG CAGATCCTCA GCGCGGATGC GTTGCCGGAT TTCGATCCCA 660

ACTTGTCGCA TGCGTTTACC AACGGCATTC TTATACGAGA GAACGGTCAC ACGATTAATC 720

CGCAAGCGCT CGTGACGCTC TTGTTTCGCC GTTTTATCGC GAACGGTCGC GAATTCGTAT 780

CTGCGCGTGT CATCGGCTTT GAGACTGAAG GTACGGCGCT TAAACGCATT ACAACCACGA 840

ACGGCGTTCT GGCGGTTGAT GCAGCGGTTG TCGCAGCGCG GGCACACTGG AAATCACTTG 900

CTAATTCGCT ACGCGATGAC ATCGCGCTGG ATACCGAACG TGGATATCAT ATCGTCATCG 960

CGAATCGCGA AGCCGCTCCA GGCATTCGGA GGACGGATGC GTCAGGAAAA TTCATGGCGA 1020

CACCTATCGA AATCGGGCTT GCGGTGGCGG GTACGGTTGA GTTCGCTGGG CTCACAGCGG 1080

CTGCTAAGTG GAAACGGTGG GATGTGCTCT ATACGCAGCC TCGAAAACTT CTTGCAGCCC 1140

TCGCGCCTGC GAGTTCTGAA GAACGATATT GCAAATCGAT GCGGTTCGCG CGGAGCATCC 1200

GCGATTCGCT CCCGGTGATT GGCGCGGCAA CGCGGACACC GGACGTAATC TATGCTTTCG 1260

GCCATGGTCA TCTGCGCATG ACAGCGGCGC CGATGACGGC AACGCTCGTC TCAGAGCTCC 1320

TCGCACGCGA AAAGACCTCA ATCGACATTT CGCGCTTCGC ACCAAACGCG TTTGCTATTG 1380

GCAAATCGCA GCAAACGCGT CGCGCAAGTT AAGTACTTAC GCCGTCGTGA GTACAGCGCA 1440

GACGCGCTGT CAAGATCAAT CTGCACCTCG CAATCACGTC GGAGACGCGA AATGCGGCAA 1500

ATAGAACACA TATTAACGAG TCACGCGCGG AACGCTTTCG GTCACTACAG TCAGCGCGCC 1560

CGAGCGCGTG GATTCATTCA TGTTTGCGGT CAGCTTCGGA TCAAACCAGA AGGCCAGTCG 1620

GAGCAATCTG ACGATCTCGT CGATAACCAG GCCAGTCTCG TTCTCCCGAA TTTGCTGGCC 1680

GTACTCGAG 1689

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1293 base pairs
(B) TYPE: nucl ic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1293

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG TCT GAG AAC CAC AAA AAA GTA GGC ATC GCT GGA GCC GGA ATC GTC      48
Met Ser Glu Asn His Lys Lys Val Gly Ile Ala Gly Ala Gly Ile Val
  1               5                  10                  15

GGC GTA TGC ACG GCG CTG ATG CTT CAG CGC CGC GGA TTC AAA GTC ACC      96
Gly Val Cys Thr Ala Leu Met Leu Gln Arg Arg Gly Phe Lys Val Thr
             20                  25                  30

TTG ATT GAC CCG AAC CCT CCT GGC GAA GGT GCA TCG TTT GGG AAT GCC     144
Leu Ile Asp Pro Asn Pro Pro Gly Glu Gly Ala Ser Phe Gly Asn Ala
         35                  40                  45

GGA TGC TTC AAC GGC TCA TCC GTC GTC CCT ATG TCC ATG CCG GGA AAC     192
Gly Cys Phe Asn Gly Ser Ser Val Val Pro Met Ser Met Pro Gly Asn
     50                  55                  60

TTG ACG AGC GTG CCG AAG TGG CTC CTT GAC CCG ATG GGC CGT TGT CAA     240
Leu Thr Ser Val Pro Lys Trp Leu Leu Asp Pro Met Gly Arg Cys Gln
 65                  70                  75                  80

TCC GGT TCA GCT ATT TCC AAC CAT CAT GCC TGG TTG ATT CGC TTT CTG     288
Ser Gly Ser Ala Ile Ser Asn His His Ala Trp Leu Ile Arg Phe Leu
                 85                  90                  95

TTA GCC GGA AGA CCA AAC AAG GTG AAG GAG CAG GCG AAA GCA CTC CGC     336
Leu Ala Gly Arg Pr  Asn Lys Val Lys Glu Gln Ala Lys Ala Leu Arg
```

100 105 110

AAT CTC ATC AAG TCC ACG GTG CCT CTG ATC AAG TCA TTG GCG GAG GAG
Asn Leu Ile Lys Ser Thr Val Pro Leu Ile Lys Ser Leu Ala Glu Glu 384

115 120 125

GCT GAT GCG AGC CAT CTG ATC CGC CAT GAA GGT CAT CTG ACC GTA TAT
Ala Asp Ala Ser His Leu Ile Arg His Glu Gly His Leu Thr Val Tyr 432

130 135 140

CGT GGA GAA GCA GAC TTC GCC AAG GAC CGC GGA GGT TGG GAA CTG CGG
Arg Gly Glu Ala Asp Phe Ala Lys Asp Arg Gly Gly Trp Glu Leu Arg 480

145 150 155 160

CGT CTC AAC GGT GTT CGC ACG CAG ATC CTC AGC GCC GAT GCG TTG CGG
Arg Leu Asn Gly Val Arg Thr Gln Ile Leu Ser Ala Asp Ala Leu Arg 528

165 170 175

GAT TTC GAT CCG AAC TTG TCG CAT GCG TTT ACC AAG GGC ATT CTT ATA
Asp Phe Asp Pro Asn Leu Ser His Ala Phe Thr Lys Gly Ile Leu Ile 576

180 185 190

GAA GAG AAC GGT CAC ACG ATT AAT CCG CAA GGG CTC GTG ACC CTC TTG
Glu Glu Asn Gly His Thr Ile Asn Pro Gln Gly Leu Val Thr Leu Leu 624

195 200 205

TTT CGG CGT TTT ATC GCG AAC GGT GGC GAA TTC GTA TCT GCG CGT GTC
Phe Arg Arg Phe Ile Ala Asn Gly Gly Glu Phe Val Ser Ala Arg Val 672

210 215 220

ATC GGC TTT GAG ACT GAA GGT AGG GCG CTT AAA GGC ATT ACA ACC ACG
Ile Gly Phe Glu Thr Glu Gly Arg Ala Leu Lys Gly Ile Thr Thr Thr 720

225 230 235 240

AAC GGC GTT CTG GCC GTT GAT GCA GCG GTT GTC GCA GCC GGC GCA CAC
Asn Gly Val Leu Ala Val Asp Ala Ala Val Val Ala Ala Gly Ala His 768

245 250 255

TCG AAA TCA CTT GCT AAT TCG CTA GGC GAT GAC ATC CCG CTC GAT ACC 816
Ser Lys Ser Leu Ala Asn Ser Leu Gly Asp Asp Ile Pro Leu Asp Thr

260 265 270

GAA CGT GGA TAT CAT ATC GTC ATC GCG AAT CCG GAA GCC GCT CCA CGC 864
Glu Arg Gly Tyr His Ile Val Ile Ala Asn Pro Glu Ala Ala Pro Arg

275 280 285

ATT CCG ACG ACC GAT GCG TCA GGA AAA TTC ATC GCG ACA CCT ATG GAA 912
Ile Pro Thr Thr Asp Ala Ser Gly Lys Phe Ile Ala Thr Pro Met Glu

290 295 300

ATG GGG CTT CGC GTG GCG GGT ACG GTT GAG TTC GCT GGG CTC ACA GCC 960
Met Gly Leu Arg Val Ala Gly Thr Val Glu Phe Ala Gly Leu Thr Ala

305 310 315 320

GCT CCT AAC TGG AAA CGT GCG CAT GTG CTC TAT ACG CAC GCT CGA AAA 1008
Ala Pro Asn Trp Lys Arg Ala His Val Leu Tyr Thr His Ala Arg Lys

325 330 335

CTT CTT CCA GCC CTC GCG CCT GCG AGT TCT GAA GAA CGA TAT TCC AAA 1056
Leu Leu Pro Ala Leu Ala Pro Ala Ser Ser Glu Glu Arg Tyr Ser Lys

340 345 350

TGG ATG GGG TTC CGG CCG AGC ATC CCG GAT TCG CTC CCC GTG ATT GGC 1104
Trp Met Gly Phe Arg Pro Ser Ile Pro Asp Ser Leu Pro Val Ile Gly

355 360 365

CGG GCA ACC CGG ACA CCC GAC GTA ATC TAT GCT TTC GGC CAT GGT CAT 1152
Arg Ala Thr Arg Thr Pro Asp Val Ile Tyr Ala Phe Gly His Gly His

370 375 380

CTC GGC ATG ACA GGG GCG CCG ATG ACC GCA ACG CTC GTC TCA GAG CTC 1200
Leu Gly Met Thr Gly Ala Pro Met Thr Ala Thr Leu Val Ser Glu Leu

385 390 395 400

CTC GCA GGC GAA AAG ACC TCA ATC GAC ATT TCG CCC TTC GCA CCA AAC 1248
Leu Ala Gly Glu Lys Thr Ser Ile Asp Ile Ser Pro Phe Ala Pro Asn
405 410 415

CGC TTT GGT ATT GGC AAA TCC AAG CAA ACG GGT CCG GCA AGT TAA 1293
Arg Phe Gly Ile Gly Lys Ser Lys Gln Thr Gly Pro Ala Ser
420 425 430

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 430 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ser Glu Asn His Lys Lys Val Gly Ile Ala Gly Ala Gly Ile Val
1 5 10 15

Gly Val Cys Thr Ala Leu Met Leu Gln Arg Arg Gly Phe Lys Val Thr
20 25 30

Leu Ile Asp Pro Asn Pro Pro Gly Glu Gly Ala Ser Phe Gly Asn Ala
35 40 45

Gly Cys Phe Asn Gly Ser Ser Val Val Pro Met Ser Met Pro Gly Asn
50 55 60

Leu Thr Ser Val Pro Lys Trp Leu Leu Asp Pro Met Gly Arg Cys Gln
65 70 75 80

Ser Gly Ser Ala Ile Ser Asn His His Ala Trp Leu Ile Arg Phe Leu
85 90 95

Leu Ala Gly Arg Pro Asn Lys Val Lys Glu Gln Ala Lys Ala Leu Arg
100 105 110

Asn Leu Ile Lys Ser Thr Val Pro Leu Ile Lys Ser Leu Ala Glu Glu
115 120 125

Ala Asp Ala Ser His Leu Ile Arg His Glu Gly His Leu Thr Val Tyr
130 135 140

Arg Gly Glu Ala Asp Phe Ala Lys Asp Arg Gly Gly Trp Glu Leu Arg
145 150 155 160

Arg Leu Asn Gly Val Arg Thr Gln Ile Leu Ser Ala Asp Ala Leu Arg
165 170 175

Asp Phe Asp Pro Asn Leu Ser His Ala Phe Thr Lys Gly Ile Leu Ile
180 185 190

Glu Glu Asn Gly His Thr Ile Asn Pro Gln Gly Leu Val Thr Leu Leu
195 200 205

Phe Arg Arg Phe Ile Ala Asn Gly Gly Glu Phe Val Ser Ala Arg Val
210 215 220

Ile Gly Phe Glu Thr Glu Gly Arg Ala Leu Lys Gly Ile Thr Thr Thr
225 230 235 240

Asn Gly Val Leu Ala Val Asp Ala Ala Val Val Ala Ala Gly Ala His
245 250 255

Ser Lys S r Leu Ala Asn Ser Leu Gly Asp Asp Ile Pro Leu Asp Thr
260 265 270

Glu Arg Gly Tyr His Ile Val Il Ala Asn Pro Glu Ala Ala Pro Arg

275 280 285

Ile Pro Thr Thr Asp Ala Ser Gly Lys Phe Ile Ala Thr Pro Met Glu

290 295 300

Met Gly Leu Arg Val Ala Gly Thr Val Glu Phe Ala Gly Leu Thr Ala

305 310 315 320

Ala Pro Asn Trp Lys Arg Ala His Val Leu Tyr Thr His Ala Arg Lys

325 330 335

Leu Leu Pro Ala Leu Ala Pro Ala Ser Ser Glu Glu Arg Tyr Ser Lys

340 345 350

Trp Met Gly Phe Arg Pro Ser Ile Pro Asp Ser Leu Pro Val Ile Gly

355 360 365

Arg Ala Thr Arg Thr Pro Asp Val Ile Tyr Ala Phe Gly His Gly His

370 375 380

Leu Gly Met Thr Gly Ala Pro Met Thr Ala Thr Leu Val Ser Glu Leu

385 390 395 400

Leu Ala Gly Glu Lys Thr Ser Ile Asp Ile Ser Pro Phe Ala Pro Asn

405 410 415

Arg Phe Gly Ile Gly Lys Ser Lys Gln Thr Gly Pro Ala Ser

420 425 430

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1296 bas pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: doubl
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
ATGGCTGAGA ACCACAAAAA AGTAGGCATC GCTGGAGCCG GAATCGTCGG CGTATGCACG     60
GCGCTGATGC TTCAGCGCCG CGGATTCAAA GTCACCTTGA TTGACCCGAA CCCTCCTGGC    120
GAAGGTGCAT CGTTTGGGAA TGCCGGATGC TTCAACGGCT CATCCGTCGT CCCTATGTCC    180
ATGCCGGGAA ACTTGACGAG CGTGCCGAAG TGGCTCCTTG ACCCGATGGG GCCGTTGTCA    240
ATCCGGTTCA GCTATTTTCC AACCATCATG CCCTGGTTGA TTCGCTTTCT GTTAGCCGGA    300
AGACCAAACA AGGTGAAGGA GCAGGCGAAA GCACTCCGCA ATCTCATCAA GTCCACGGTG    360
CCTCTGATCA AGTCATTGGC GGAGGAGGCT GATGCGAGCC ATCTGATCCG CCATGAAGGT    420
CATCTGACCG TATATCGTGG AGAAGCAGAC TTCGCCAAGG ACCGCGGAGG TTGGGAACTG    480
CGGCGTCTCA ACGGTGTTCG CACGCAGATC CTCAGCGCCG ATGCGTTGCG GGATTTCGAT    540
CCGAACTTGT CGCATGCGTT TACCAAGGGC ATTCTTATAG AAGAGAACGG TCACACGATT    600
AATCCGCAAG GGCTCGTGAC CCTCTTGTTT CGGCGTTTTA TCGCGAACGG TGGCGAATTT    660
GTATCTGCGC GTGTCATCGG CTTTGAGACT GAAGGTAGGG CGCTTAAAGG CATTACAACC    720
ACGAACGGCG TTCTGGCCGT TGATGCAGCG GTTGTCGCAG CCGGCGCACA CTCGAAATCA    780
CTTGCTAATT CGCTAGGCGA TGACATCCCG CTCGATACCG AACGTGGATA TCATATCGTC    840
ATCGCGAATC CGGAAGCCGC TCCACGCATT CCGACGACCG ATGCGTCAGG AAAATTCATC    900
GCGACACCTA TGGAAATGGG GCTTCGCGTG GCGGGTACGG TTGAGTTCGC TGGGCTCACA    960
GCCGCTCCTA ACTGGAAACG TGCGCATGTG CTCTATACGC ACGCTCGAAA ACTTCTTCCA   1020
GCCCTCGCGC CTGCGAGTTC TGAAGAACGA TATTCCAAAT GGATGGGGTT CCGGCCGAGC   1080
ATCCCGGATT CGCTCCCCGT GATTGGCCGG GCAACCCGGA CACCCGACGT AATCTATGCT   1140
TTCGGCCACG GTCATCTCGG CATGACAGGG GCGCCGATGA CCGCAACGCT CGTCTCAGAG   1200
CTCCTCGCAG GCGAAAAGAC CTCAATCGAC ATTTCGCCCT TCGCACCAAA CCGCTTTGGT   1260
ATTGGCAAAT CCAAGCAAAC GGGTCCGGCA AGTTAA                             1296
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGGCTGAGA ACCACAAAAA AGTAGGCATC GCTGGAGCTG GAATCGTTGG TGTATGCACT     60
GCTTTGATGC TTCAACGTCG TGGATTCAAA GTCACCTTGA TTGACCCGAA CCCTCCTGGC    120
GAAGGTGCAT CGTTTGGGAA TGCCGGATGC TTCAACGGCT CATCCGTCGT CCCTATGTCC    180
ATGCCGGGAA ACTTGACGAG CGTGCCGAAG TGGCTCCTTG ACCCGATGGG GCCGTTGTCA    240
ATCCGGTTCA GCTATTTTCC AACCATCATG CCCTGGTTGA TTCGCTTTCT GTTAGCCGGA    300
AGACCAAACA AGGTGAAGGA GCAGGCGAAA GCACTCCGCA ATCTCATCAA GTCCACGGTG    360
CCTCTGATCA AGTCATTGGC GGAGGAGGCT GATGCGAGCC ATCTGATCCG CCATGAAGGT    420
CATCTGACCG TATATCGTGG AGAAGCAGAC TTCGCCAAGG ACCGCGGAGG TTGGGAACTG    480
CGGCGTCTCA ACGGTGTTCG CACGCAGATC CTCTCTGCTG ATGCTTTGCG TGATTTCGAT    540
CCTAACTTGT CGCATGCTTT TACCAAGGGC ATTCTTATAG AAGAGAACGG TCACACGATT    600
AATCCGCAAG GGCTCGTGAC CCTCTTGTTT CGGCGTTTTA TCGCGAACGG TGGCGAATTT    660
GTATCTGCGC GTGTCATCGG TTTTGAGACT GAAGGTCGTG CTCTCAAAGG CATTACAACC    720
ACTAACGGTG TTCTGGCTGT TGATGCAGCT GTTGTTGCAG CTGGTGCACA CTCTAAATCA    780
CTTGCTAATT CGCTAGGCGA TGACATCCCG CTCGATACCG AACGTGGATA TCATATCGTC    840
ATCGCGAATC CGGAAGCCGC TCCACGCATT CCGACGACCG ATGCGTCAGG AAAATTCATC    900
GCGACACCTA TGGAAATGGG TCTTCGTGTT GCTGGTACTG TTGAGTTTGC TGGTCTCACA    960
GCTGCTCCTA ACTGGAAACG TGCGCATGTG CTCTATACGC ACGCTCGAAA ACTTCTTCCA   1020
GCCCTCGCGC CTGCGAGTTC TGAAGAACGA TATTCCAAAT GGATGGGTTT TCGTCCTAGC   1080
ATTCCTGATT CTCTTCCAGT GATTGGTCGT GCAACTCGTA CACCCGACGT AATCTATGCT   1140
TTTGGTCACG GTCATCTCGG TATGACAGGT GCTCCAATGA CTGCAACTCT CGTCTCAGAG   1200
```

CTCCTCGCAG GCGAAAAGAC CTCAATCGAC ATTTCGCCCT TCGCACCAAA CCGCTTTGGT 1260

ATTGGCAAAT CCAAGCAAAC GGGTCCGGCA AGTTAA 1296

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGGCTGAGA ACCACAAGAA GGTTGGTATC GCTGGAGCTG GAATCGTTGG TGTTTGCACT 60

GCTTTGATGC TTCAACGTCG TGGATTCAAG GTTACCTTGA TTGATCCAAA CCCACCAGGT 120

GAAGGTGCTT CTTTCGGTAA CGCTGGTTGC TTCAACGGTT CCTCCGTTGT TCCAATGTCC 180

ATGCCAGGAA ACTTGACTAG CGTTCCAAAG TGGCTTCTTG ACCCAATGGG TCCATTGTCC 240

ATCCGTTTCA GCTACTTTCC AACCATCATG CCTTGGTTGA TTCGTTTCTT GCTTGCTGGA 300

AGACCAAACA AGGTGAAGGA GCAAGCTAAG GCACTCCGTA ACCTCATCAA GTCCACTGTG 360

CCTTTGATCA AGTCCTTGGC TGAGGAGGCT GATGCTAGCC ACCTTATCCG TCACGAAGGT 420

CACCTTACCG TGTACCGTGG AGAAGCAGAC TTCGCCAAGG ACCGTGGAGG TTGGGAACTT 480

CGTCGTCTCA ACGGTGTTCG TACTCAAATC CTCAGCGCTG ATGCATTGCG TGATTTCGAT 540

CCTAACTTGT CTCACGCCTT TACCAAGGGA ATCCTTATCG AAGAGAACGG TCACACCATC 600

AACCCACAAG GTCTCGTGAC TCTCTTGTTT CGTCGTTTCA TCGCTAACGG TGGAGAGTTC 660

GTGTCTGCTC GTGTTATCGG ATTCGAGACT GAAGGTCGTG CTCTCAAGGG TATCACCACC 720

ACCAACGGTG TTCTTGCTGT TGATGCAGCT GTTGTTGCAG CTGGTGCACA CTCCAAGTCT 780

CTTGCTAACT CCCTTGGTGA TGACATCCCA TTGGATACCG AACGTGGATA CCACATCGTG 840

ATCGCCAACC CAGAAGCTGC TCCACGTATT CCAACTACCG ATGCTTCTGG AAAGTTCATC 900

GCTACTCCTA TGGAGATGGG TCTTCGTGTT GCTGGAACCG TTGAGTTCGC TGGTCTCACT 960

GCTGCTCCTA ACTGGAAGCG TGCTCACGTT CTCTACACTC ACGCTCGTAA GTTGCTTCCA 1020

GCTCTCGCTC CTGCCAGTTC TGAAGAACGT TACTCCAAGT GGATGGGTTT CCGTCCAAGC 1080

ATCCCAGATT CCCTTCCAGT GATTGGTCGT GCTACCCGTA CTCCAGACGT TATCTACGCT 1140

TTCGGTCACG TCACCTCGG TATGACTGGT GCTCCAATGA CCGCAACCCT CGTTTCTGAG 1200

CTCCTCGCAG GTGAGAAGAC CTCTATCGAC ATCTCTCCAT TCGCACCAAA CCGTTTCGGT 1260

ATTGGTAAGT CCAAGCAAAC TGGTCCTGCA TCCTAA 1296

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 279 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGATCTCCAC AATGGCTTCC TCTATGCTCT CTTCCGCTAC TATGGTTGCC TCTCCGGCTC 60

AGGCCACTAT GGTCGCTCCT TTCAACGGAC TTAAGTCCTC CGCTGCCTTC CCAGCCACCC 120

GCAAGGCTAA CAACGACATT ACTTCCATCA CAAGCAACGG CGGAAGAGTT AACTGCATGC 180

AGGTGTGGCC TCCGATTGGA AAGAAGAAGT TTGAGACTCT CTCTTACCTT CCTGACCTTA 240

CCGATTCCGG TGGTCGCGTC AACTGCATGC AGGCCATGG 279

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGATCTATCG ATAAGCTTGA TGTAATTGGA GGAAGATCAA AATTTTCAAT CCCCATTCTT 60

CGATTGCTTC AATTGAAGTT TCTCCGATGG CGCAAGTTAG CAGAATCTGC AATGGTGTGC 120

AGAACCCATC TCTTATCTCC AATCTCTCGA AATCCAGTCA ACGCAAATCT CCCTTATCGG 180

TTTCTCTGAA GACGCAGCAG CATCCACGAG CTTATCCGAT TTCGTCGTCG TGGGGATTGA 240

AGAAGAGTGG GATGACGTTA ATTGGCTCTG AGCTTCGTCC TCTTAAGGTC ATGTCTTCTG 300

TTTCCACGGC GTGCATGC 318

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

NCATGGACGT CTGATCGAAA TCGTCGTTAC CGCAGCAAGG TAAGGCACGC CGAATTTTAT 60

CACCTACCGC GAAACGGTGG CTAGGCAGCG AGAGACTGTC GGCTCCGCGG GAGCATCCT 119

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTACTTACGC GGTCGTGAGT ACAGCGCAGA GCCGGTGTCA AGATCAATCT GCACCTCGCA 60

ATCACCTCGG AGACGCGAAA TGGCGCAAAT AGAACACATA TTAACGAGTC ACGCCCCGAA 120

GCCTTTGGGT CACTACAGTC AGGCGGCCCG AGCGGGTGGA TTCATTCATG TTTCCGGTCA 180

GCTTCCGATC AAACCAGAAG GCCAGTCGGA GCAATCTGAC GATCTCGTCG ATAACCAGGC 240

CAGTCTCGTT CTCCGGAATT TGCTGGCCGT ACTCGAG 277

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGAGACTGT CGACTCCGCG GGAGCATCAT ATG 33

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAACGAATCC AAGCTTCTCA CGACCGCGTA AGTAC 35

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCGAGATGA CCGTGGCCGA AAGC 24

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGGAATGCCG GATGCTTCAA CGGC 24

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (recombinant)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1296

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG GCT GAG AAC CAC AAG AAG GTT GGT ATC GCT GGA GCT GGA ATC GTT 48
Met Ala Glu Asn His Lys Lys Val Gly Ile Ala Gly Ala Gly Ile Val
1 5 10 15

GGT GTT TGC ACT GCT TTG ATG CTT CAA CGT CGT GGA TTC AAG GTT ACC 96
Gly Val Cys Thr Ala Leu Met Leu Gln Arg Arg Gly Phe Lys Val Thr
20 25 30

TTG ATT GAT CCA AAC CCA CCA GGT GAA GGT GCC TCT TTC GGT AAC GCT 144
Leu Ile Asp Pro Asn Pro Pro Gly Glu Gly Ala Ser Phe Gly Asn Ala
35 40 45

GGT TGC TTC AAC GGT TCC TCC GTT GTT CCA ATG TCC ATG CCA GGA AAC 192
Gly Cys Phe Asn Gly Ser Ser Val Val Pro Met Ser Met Pro Gly Asn
50 55 60

TTG ACT AGC GTT CCA AAG TGG CTT CTT GAC CCA ATG GGT CCA TTG TCC 240
Leu Thr Ser Val Pro Lys Trp Leu Leu Asp Pro Met Gly Pro Leu Ser
65 70 75 80

ATC CGT TTC GGC TAC TTT CCA ACC ATC ATG CCT TGG TTG ATT CGT TTC 288
Ile Arg Phe Gly Tyr Phe Pro Thr Ile Met Pro Trp Leu Ile Arg Phe
85 90 95

TTG CTT GCT GGA AGA CCA AAC AAG GTG AAG GAG CAA GCT AAG GCA CTC 336
Leu Leu Ala Gly Arg Pro Asn Lys Val Lys Glu Gln Ala Lys Ala Leu

100 105 110

CGT AAC CTC ATC AAG TCC ACT GTG CCT TTG ATC AAG TCC TTG GCT GAG 384
Arg Asn Leu Ile Lys Ser Thr Val Pro Leu Ile Lys Ser Leu Ala Glu

115 120 125

GAG GCT GAT GCT AGC CAC CTT ATC CGT CAC GAA GGT CAC CTT ACC GTG 432
Glu Ala Asp Ala Ser His Leu Ile Arg His Glu Gly His Leu Thr Val

130 135 140

TAC CGT GGA GAA GCA GAC TTC GCC AGG GAC CGT GGA GGT TGG GAA CTT 480
Tyr Arg Gly Glu Ala Asp Phe Ala Arg Asp Arg Gly Gly Trp Glu Leu

145 150 155 160

CGT CGT CTC AAC GGT GTT CGT ACT CAA ATC CTC AGC GCT GAT GCA TTG 528
Arg Arg Leu Asn Gly Val Arg Thr Gln Ile Leu Ser Ala Asp Ala Leu

165 170 175

CGT GAT TTC GAT CCT AAC TTG TCT CAC GCC TTT ACC AAG GGA ATC CTT 576
Arg Asp Phe Asp Pro Asn Leu Ser His Ala Phe Thr Lys Gly Ile Leu

180 185 190

ATC GAA GAG AAC GGT CAC ACC ATC AAC CCA CAA GGT CTC GTG ACT CTC 624
Ile Glu Glu Asn Gly His Thr Ile Asn Pro Gln Gly Leu Val Thr Leu

195 200 205

TTG TTT CGT CGT TTC ATC GCT AAC GGT GGA GAG TTC GTG TCT GCT CGT 672
Leu Phe Arg Arg Phe Ile Ala Asn Gly Gly Glu Phe Val Ser Ala Arg

210 215 220

GTT ATC GGA TTC GAG ACT GAA GGT CGT GCT CTC AAG GGT ATC ACC ACC 720
Val Ile Gly Phe Glu Thr Glu Gly Arg Ala Leu Lys Gly Ile Thr Thr

225 230 235 240

ACC AAC GGT GTT CTT GCT GTT GAT GCA GCT GTT GTT GCA GCT GGT GCA 768
Thr Asn Gly Val L u Ala Val Asp Ala Ala Val Val Ala Ala Gly Ala
245 250 255

CAC TCC AAG TCT CTT GCT AAC TCC CTT GGT GAT GAC ATC CCA TTG GAT 816
His Ser Lys Ser Leu Ala Asn Ser Leu Gly Asp Asp Ile Pro Leu Asp
260 265 270

ACC GAA CGT GGA TAC CAC ATC GTG ATC GCC AAC CCA GAA GCT GCT CCA 864
Thr Glu Arg Gly Tyr His Ile Val Ile Ala Asn Pro Glu Ala Ala Pro
275 280 285

CGT ATT CCA ACT ACC GAT GCT TCT GGA AAG TTC ATC GCT ACT CCT ATG 912
Arg Ile Pro Thr Thr Asp Ala Ser Gly Lys Phe Ile Ala Thr Pro Met
290 295 300

GAG ATG GGT CTT CGT GTT GCT GGA ACC GTT GAG TTC GCT GGT CTC ACT 960
Glu Met Gly Leu Arg Val Ala Gly Thr Val Glu Phe Ala Gly Leu Thr
305 310 315 320

GCT GCT CCT AAC TGG AAG CGT GCT CAC GTT CTC TAC ACT CGC GCT CGT 1008
Ala Ala Pro Asn Trp Lys Arg Ala His Val Leu Tyr Thr Arg Ala Arg
325 330 335

AAG TTG CTT CCA GCT CTC GCT CCT GCC AGT TCT GAA GAA CGT TAC TCC 1056
Lys Leu Leu Pro Ala Leu Ala Pro Ala Ser Ser Glu Glu Arg Tyr Ser
340 345 350

AAG TGG ATG GGT TTC CGT CCA AGC ATC CCG GAT TCC CTT CCA GTG ATT 1104
Lys Trp Met Gly Phe Arg Pro Ser Ile Pro Asp Ser Leu Pro Val Ile
355 360 365

GGT CGT GCT ACC CGT ACT CCA GAC GTT ATC TAC GCT TTC GGT CAC GGT 1152
Gly Arg Ala Thr Arg Thr Pro Asp Val Ile Tyr Ala Phe Gly His Gly
370 375 380

CAC CTC GGT ATG ACT GGT GCT CCA ATG ACC GCA ACC CTC GTT TCT GAG 1200
His Leu Gly Met Thr ly Ala Pro Met Thr Ala Thr Leu Val Ser Glu
385 390 395 400

CTC CTC GCA GGT GAG AAG ACC TCT ATC GAC ATC TCT CCA TTC GCA CCA 1248
Leu Leu Ala Gly Glu Lys Thr Ser Ile Asp Ile Ser Pro Phe Ala Pro
405 410 415

AAC CGT TTC GGT ATT GGT AAG TCC AAG CAA ACT GGT CCT GCA TCC TAA 1296
Asn Arg Phe Gly Ile Gly Lys Ser Lys Gln Thr Gly Pro Ala Ser
420 425 430

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 431 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Ala Glu Asn His Lys Lys Val Gly Ile Ala Gly Ala Gly Ile Val
1 5 10 15

Gly Val Cys Thr Ala Leu Met Leu Gln Arg Arg Gly Phe Lys Val Thr
20 25 30

Leu Ile Asp Pro Asn Pro Pro Gly Glu Gly Ala Ser Phe Gly Asn Ala
35 40 45

Gly Cys Phe Asn Gly Ser Ser Val Val Pro Met Ser Met Pro Gly Asn
50 55 60

Leu Thr Ser Val Pro Lys Trp Leu Leu Asp Pro Met Gly Pro Leu Ser
65 70 75 80

Ile Arg Phe Gly Tyr Phe Pro Thr Ile Met Pro Trp Leu Ile Arg Phe

85 90 95

Leu Leu Ala Gly Arg Pro Asn Lys Val Lys Glu Gln Ala Lys Ala Leu

100 105 110

Arg Asn Leu Ile Lys Ser Thr Val Pro Leu Ile Lys Ser Leu Ala Glu

115 120 125

Glu Ala Asp Ala Ser His Leu Ile Arg His Glu Gly His Leu Thr Val

130 135 140

Tyr Arg Gly Glu Ala Asp Phe Ala Arg Asp Arg Gly Gly Trp Glu Leu

145 150 155 160

Arg Arg Leu Asn Gly Val Arg Thr Gln Ile Leu Ser Ala Asp Ala Leu

165 170 175

Arg Asp Phe Asp Pro Asn Leu Ser His Ala Phe Thr Lys Gly Ile Leu

180 185 190

Ile Glu Glu Asn Gly His Thr Ile Asn Pro Gln Gly Leu Val Thr Leu

195 200 205

Leu Phe Arg Arg Phe Ile Ala Asn Gly Gly Glu Phe Val Ser Ala Arg

210 215 220

Val Ile Gly Phe Glu Thr Glu Gly Arg Ala Leu Lys Gly Ile Thr Thr

225 230 235 240

Thr Asn Gly Val Leu Ala Val Asp Ala Ala Val Val Ala Ala Gly Ala

245 250 255

His Ser Lys Ser L u Ala Asn Ser Leu Gly Asp Asp Ile Pro Leu Asp

260 265 270

Thr Glu Arg Gly Tyr His Ile Val Ile Ala Asn Pro Glu Ala Ala Pro

275 280 285

Arg Ile Pro Thr Thr Asp Ala Ser Gly Lys Phe Ile Ala Thr Pro Met

290 295 300

Glu Met Gly Leu Arg Val Ala Gly Thr Val Glu Phe Ala Gly Leu Thr

305 310 315 320

Ala Ala Pro Asn Trp Lys Arg Ala His Val Leu Tyr Thr Arg Ala Arg

325 330 335

Lys Leu Leu Pro Ala Leu Ala Pro Ala Ser Ser Glu Glu Arg Tyr Ser

340 345 350

Lys Trp Met Gly Phe Arg Pro Ser Ile Pro Asp Ser Leu Pro Val Ile

355 360 365

Gly Arg Ala Thr Arg Thr Pro Asp Val Ile Tyr Ala Phe Gly His Gly

370 375 380

His Leu Gly Met Thr Gly Ala Pro Met Thr Ala Thr Leu Val Ser Glu

385 390 395 400

Leu Leu Ala Gly Glu Lys Thr Ser Ile Asp Ile Ser Pro Phe Ala Pro

405 410 415

Asn Arg Phe Gly Ile Gly Lys S r Lys Gln Thr Gly Pro Ala S r

420 425 430

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGTTCTCTAC ACTCGTGCTC GTAAGTTGC
29

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGTTCTCTAC ACTAAGGCTC GTAAGTTGC 29

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGTTCTCTAC ACTCAAGCTC GTAAGTTGC 29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGTTCTCTAC ACTGCTGCTC GTAAGTTGC 29

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCTACACTT GGGCTCGTAA GCTTCTTCCA GC 32

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCTACACTA TCGCTCGTAA GCTTCTTCCA GC 32

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTCTACACTC TGGCTCGTAA GCTTCTTCCA GC 32

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTCTACACTG AAGCTCGTAA GCTTCTTCCA GC 32

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CGCTGGAGCT GGAATCGTTG GTGTATGCAC TGCTTTGATG CTTCAACGTC GTGGATTCAA 60

AG 62

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCAGATCCTC TCTGCTGATG CTTTGCGTGA TTTCGATCCT AACTTGTCTC ATGCTTTTAC 60

CAAGG 65

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GTCATCGGTT TTGAGACTGA AGGTCGTGCT CTCAAAGGCA T 41

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TACAACCACT AACGGTGTTC TGGCTGTTGA TGCAGCTGTT GTTGCAGCTG GTGCACACTC 60
TAAATCACT 69

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGAAATGGGT CTTCGTGTTG CTGGTACTGT TGAGTTTGCT GGTCTCACAG CTGCTCCTAA 60

C 61

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TGGATGGGTT TTCGTCCTAG CATTCCTGAT TCTCTTCCAG TGATTGGTCG TGCAACTCGT 60

ACACCCGA 68

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CGTAATCTAT GCTTTTGGTC ACGGTCATCT CGGTATGACA GGTGCTCCAA TGACTGCAAC 60

TCTCGTCTC 69

Claims:

1. An isolated double-stranded DNA molecule consisting essentially of DNA encoding a glyphosate oxidoreductase enzyme.

2. A recombinant, double-stranded DNA molecule comprising in sequence:

a) a promoter which functions in plants to cause the production of an RNA sequence;

b) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxidoreductase enzyme; and

c) a 3' non-translated region which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence

where the promoter is heterologous with respect to the structural DNA sequence and adapted to cause sufficient expression of said enzyme in plant tissue, including meristematic tissue, to enhance the glyphosate tolerance of a plant cell transformed with said gene.

3. A DNA molecule of Claim 2 in which said structural DNA sequence encodes a fusion polypeptide comprising an amino-terminal chloroplast transit peptide and a glyphosate oxidoreductase enzyme.

4. A DNA molecule of Claim 3 in which the promoter is a plant DNA virus promoter.

5. A DNA molecule of Claim 4 in which the promoter is selected from the group consisting of CaMV35S and FMV35S promoters.

6. A method of producing genetically transformed plants which are tolerant toward glyphosate herbicide, comprising the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising:

   i) a promoter which functions in plant cells to cause the production of an RNA sequence,

   ii) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxido-reductase enzyme,

   iii) a 3' non-translated DNA sequence which functions in plant cells to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence

   where the promoter is heterologous with respect to the structural DNA sequence and adapted to cause sufficient expression of said enzyme in plant tissue, including meristematic tissue, to enhance the glyphosate tolerance of a plant cell transformed with said gene;

b) obtaining a transformed plant cell; and

c) regenerating from the transformed plant cell a genetically transformed plant which has increased tolerance to glyphosate herbicide.

7. A method of Claim 6 in which said structural DNA sequence encodes a fusion polypeptide comprising an amino terminal chloroplast transit peptide and a glyphosate oxidoreductase enzyme.

8. A method of Claim 7 in which the promoter is from a plant DNA virus.

9. A method of Claim 8 in which the promoter is selected from the group consisting of CaMV35S and FMV35S promoters.

10. A glyphosate tolerant plant cell comprising a DNA molecule of Claim 3.

11. A glyphosate tolerant plant cell of Claim 10 in which the promoter is a plant DNA virus promoter.

12. A glyphosate tolerant plant cell of Claim 11 in which the promoter is selected from the group consisting of CaMV35S and FMV35S promoters.

13. A glyphosate tolerant plant cell of Claim 10 selected from the group consisting of corn, wheat, rice, soybean, cotton, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tobacco, tomato, alfalfa, lettuce, apple, poplar and pine.

14. A glyphosate tolerant plant comprising plant cells of Claim 10.

15. A glyphosate tolerant plant of Claim 14 in which the promoter is from a DNA plant virus promoter.

16. A glyphosate tolerant plant of Claim 15 in which the promoter is selected from the group consisting of CaMV35S and FMV35S promoters.

17. A glyphosate tolerant plant of Claim 14 selected from the group consisting of corn, wheat, rice, soybean, cotton, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tobacco, tomato, alfalfa, lettuce, apple, poplar and pine.

18. A method for selectively controlling weeds in a field containing a crop having planted crop seeds or plants comprising the steps of:

a) planting said crop seeds or plants which are glyphosate tolerant as a result of a recombinant double-stranded DNA molecule being inserted into said crop seed or plant, said DNA molecule having:

i) a promoter which functions in plants to cause the production of an RNA sequence,

ii) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxido-reductase enzyme,

iii) a 3' non-translated DNA sequence which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence

where the promoter is heterologous with respect to the structural DNA sequence and adapted to cause sufficient expression of said enzym in plant tissue, including

meristematic tissue to enhance the glyphosate tolerance of a plant transformed with said gene; and

b) applying to said crop and weeds in said field a sufficient amount of glyphosate herbicide to control said weeds without significantly affecting said crop.

19. A method of Claim 18 in which said structural DNA sequence encodes an amino terminal chloroplast transit peptide and a glyphosate oxidoreductase enzyme.

20. A method of Claim 19 in which the crop plant is selected from the group consisting of corn, wheat, rice, soybean, cotton, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tobacco, tomato, lettuce, apple, poplar, pine and alfalfa.

21. A DNA of Claim 1 which hybridizes to the DNA sequence of SEQ ID NO:3.

22. A glyphosate oxidoreductase protein substantially free of other bacterial proteins comprising the amino acid sequence as set forth in SEQ ID NO:5.

23. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is arginine.

24. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is lysine.

25. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is glutamine.

26. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is alanine.

27. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is tryptophan.

28. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is isoleucine.

29. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is leucine.

30. The glyphosate oxidoreductase protein of claim 22 wherein the amino acid residue at position 334 is glutamic acid.

31. A method for selecting transformed plant tissue comprising:

introducing a gene encoding glyphosate oxidoreductase into plant tissue;

placing said plant tissue on a plant growth media containing glyphosate;

selecting plant tissue which exhibits growth on said glyphosate containing media.

32. The method of claim 31 further comprising the step of confirming the presence of said glyphosate oxidoreductase gene in said plant tissue by recallusing on glyphosate a segment of said plant tissue exhibiting growth on glyphosate containing media.

33. A recombinant bacterium containing the DNA of claim 1.

34. A glyphosate oxidoreductase enzyme catalyzing the oxidation of glyphosate to aminomethylphosphonate and glyoxylate.

SspI
6358 TCATCAAAATATTTAGCAGCATTCCAGATTGGGTTCAA

TCAACAAGGTACGAGCCATATCACTTTATTCAAATTGG

TATCGCCAAAACCAAGAAGGAACTCCCATCCTCAAAGG

TTTGTAAGGAAGAATTCTCAGTCCAAAGCCTCAACAAG

GTCAGGGTACAGAGTCTCCAAACCATTAGCCAAAAGCT

ACAGGAGATCAATGAAGAATCTTCAATCAAAGTAAACT

ACTGTTCCAGCACATGCATCATGGTCAGTAAGTTTCAG

AAAAAGACATCCACCGAAGACTTAAAGTTAGTGGGCAT

CTTTGAAAGTAATCTTGTCAACATCGAGCAGCTGGCTT

GTGGGGACCAGACAAAAAAGGAATGGTGCAGAATTGTT

AGGCGCACCTACCAAAAGCATCTTTGCCTTTATTGCAA

AAGATAAAGCAGATTCCTCTAGTACAAGTGGGGAACAA

AATAACGTGGAAAAGAGCTGTCCTGACAGCCCACTCAC

TAATGCGTATGACGAACGCAGTGACGACCACAAAAGAA

TTTTCCCTCTATATAAGAAGGCATTTCATTCCCATTTG

AAGGATCATCAGATACTAACCAATATTTCTC 6954
SspI

FIG.1

```
  1  NCATGGACGTCTGATCGAAATCGTCGTTACCGCAGCAAGGTAAGGCACGCCGAATTTTAT
 61  CACCTACCGCGAAACGGTGGCTAGGCAGCGAGAGACTGTCGGCTCCGCGGGAGCATCCTA
                                                               M ('Met120')
121  TGTCTGAGAACCACAAAAAAGTAGGCATCGCTGGAGCCGGAATCGTCGGCGTATGCACGG
       S  E  N  H  K  K  V  G  I  A  G  A  G  I  V  G  V  C  T  A
181  CGCTGATGCTTCAGCGCCGCGGATTCAAAGTCACCTTGATTGACCCGAACCCTCCTGGCG
       L  M  L  Q  R  R  G  F  K  V  T  L  I  D  P  N  P  P  G  E
241  AAGGTGCATCGTTTGGGAATGCCGGATGCTTCAACGGCTCATCCGTCGTCCCTATGTCCA
       G  A  S  F  G  N  A  G  C  F  N  G  S  S  V  V  P  M  S  M
301  TGCCGGGAAACTTGACGAGCGTGCCGAAGTGGCTCCTTGACCCGATGGGGCCGTTGTCAA
       P  G  N  L  T  S  V  P  K  W  L  L  D  P  M  G  P  L  S  I
361  TCCGGTTCAGCTATTTTCCAACCATCATGCCCTGGTTGATTCGCTTTCTGTTAGCCGGAA
       R  F  S  Y  F  P  T  I  M  P  W  L  I  R  F  L  L  A  G  R
421  GACCAAACAAGGTGAAGGAGCAGGCGAAAGCACTCCGCAATCTCATCAAGTCCACGGTGC
       P  N  K  V  K  E  Q  A  K  A  L  R  N  L  I  K  S  T  V  P
481  CTCTGATCAAGTCATTGGCGGAGGAGGCTGATGCGAGCCATCTGATCCGCCATGAAGGTC
       L  I  K  S  L  A  E  E  A  D  A  S  H  L  I  R  H  E  G  H
541  ATCTGACCGTATATCGTGGAGAAGCAGACTTCGCCAAGGACCGCGGAGGTTGGGAACTGC
       L  T  V  Y  R  G  E  A  D  F  A  K  D  R  G  G  W  E  L  R
601  GGCGTCTCAACGGTGTTCGCACGCAGATCCTCAGCGCCGATGCGTTGCGGGATTTCGATC
       R  L  N  G  V  R  T  Q  I  L  S  A  D  A  L  R  D  F  D  P
                 SphI
661  CGAACTTGTCGCATGCGTTTACCAAGGGCATTCTTATAGAAGAGAACGGTCACACGATTA
       N  L  S  H  A  F  T  K  G  I  L  I  E  E  N  G  H  T  I  N
                                                          EcoRI
721  ATCCGCAAGGGCTCGTGACCCTCTTGTTTCGGCGTTTTATCGCGAACGGTGGCGAATTCG
       P  Q  G  L  V  T  L  L  F  R  R  F  I  A  N  G  G  E  F  V
781  TATCTGCGCGTGTCATCGGCTTTGAGACTGAAGGTAGGGCGCTTAAAGGCATTACAACCA
       S  A  R  V  I  G  F  E  T  E  G  R  A  L  K  G  I  T  T  T
841  CGAACGGCGTTCTGGCCGTTGATGCAGCGGTTGTCGCAGCCGGCGCACACTCGAAATCAT
       N  G  V  L  A  V  D  A  A  V  V  A  A  G  A  H  S  K  S  L
                                                    EcoRV
```

FIG.2A

```
901   TTGCTAATTCGCTAGGCGATGACATCCCGCTCGATACCGAACGTGGATATCATATCGTCA
        A  N  S  L  G  D  D  I  P  L  D  T  E  R  G  Y  H  I  V  I
961   TCGCGAATCCGGAAGCCGCTCCACGCATTCCGACGACCGATGCGTCAGGAAAATTCATCG
        A  N  P  E  A  A  P  R  I  P  T  T  D  A  S  G  K  F  I  A
1021  CGACACCTATGGAAATGGGGCTTCGCGTGGCGGGTACGGTTGAGTTCGCTGGGCTCACAG
        T  P  M  E  M  G  L  R  V  A  G  T  V  E  F  A  G  L  T  A
1081  CCGCTCCTAACTGGAAACGTGCGCATGTGCTCTATACGCACGCTCGAAAACTTCTTCCAG
        A  P  N  W  K  R  A  H  V  L  Y  T  H  A  R  K  L  L  P  A
1141  CCCTCGCGCCTGCGAGTTCTGAAGAACGATATTCCAAATGGATGGGGTTCCGGCCGAGCA
        L  A  P  A  S  S  E  E  R  Y  S  K  W  M  G  F  R  P  S  I
1201  TCCCGGATTCGCTCCCCGTGATTGGCCGGGCAACCCGGACACCCGACGTAATCTATGCTT
        P  D  S  L  P  V  I  G  R  A  T  R  T  P  D  V  I  Y  A  F
         NcoI                                                  SacI
1261  TCGGCCATGGTCATCTCGGCATGACAGGGGCGCCGATGACCGCAACGCTCGTCTCAGAGC
        G  H  G  H  L  G  M  T  G  A  P  M  T  A  T  L  V  S  E  L
1321  TCCTCGCAGGCGAAAAGACCTCAATCGACATTTCGCCCTTCGCACCAAACCGCTTTGGTA
        L  A  G  E  K  T  S  I  D  I  S  P  F  A  P  N  R  F  G  I
                                              ScaI
1381  TTGGCAAATCCAAGCAAACGGGTCCGGCAAGTTAAGTACTTACGCGGTCGTGAGTACAGC
        G  K  S  K  Q  T  G  P  A  S  ***
1441  GCAGAGCCGGTGTCAAGATCAATCTGCACCTCGCAATCACCTCGGAGACGCGAAATGGCG
1501  CAAATAGAACACATATTAACGAGTCACGCCCCGAAGCCTTTGGGTCACTACAGTCAGGCG
1561  GCCCGAGCGGGTGGATTCATTCATGTTTCCGGTCAGCTTCCGATCAAACCAGAAGGCCAG
1621  TCGGAGCAATCTGACGATCTCGTCGATAACCAGGCCAGTCTCGTTCTCCGGAATTTGCTG
          XhoI
1681  GCCGTACTCGAG
```

FIG.2B

```
            fMet
  1  AGATCTCCATGGCTGAGAACCACAAAAAAGTAGGCATCGCTGGAGCCGGA   50
                                                   T
 51  ATCGTCGGCGTATGCACGGCGCTGATGCTTCAGCGCCGCGGATTCAAAGT  100
          T  T        T  TT          A  T  T
101  CACCTTGATTGACCCGAACCCTCCTGGCGAAGGTGCATCGTTTGGGAATG  150
151  CCGGATGCTTCAACGGCTCATCCGTCGTCCCTATGTCCATGCCGGGAAAC  200
201  TTGACGAGCGTGCCGAAGTGGCTCCTTGACCCGATGGGGCCGTTGTCAAT  250
251  CCGGTTCAGCTATTTTCCAACCATCATGCCCTGGTTGATTCGCTTTCTGT  300
301  TAGCCGGAAGACCAAACAAGGTGAAGGAGCAGGCGAAAGCACTCCGCAAT  350
351  CTCATCAAGTCCACGGTGCCTCTGATCAAGTCATTGGCGGAGGAGGCTGA  400
401  TGCGAGCCATCTGATCCGCCATGAAGGTCATCTGACCGTATATCGTGGAG  450
451  AAGCAGACTTCGCCAAGGACCGCGGAGGTTGGCAACTGCGGCGTCTCAAC  500
501  GGTGTTCGCACGCAGATCCTCAGCGCCGATGCGTTGCGGGATTTCGATCC  550
                          TCT  T     T     T
551  GAACTTGTCGCATGCGTTTACCAAGGGCATTCTTATAGAAGAGAACGGTC  600
     T              T
```

FIG.3A

```
601   ACACGATTAATCCGCAAGGGCTCGTGACCCTCTTGTTTCGGCGTTTTATC   650

651   GCGAACGGTGGCGAATTTGTATCTGCGCGTGTCATCGGCTTTGAGACTGA   700
                                            T

701   AGGTAGGGCGCTTAAAGGCATTACAACCACGAACGGCGTTCTGGCCGTTG   750
          C T  T  C                 T     T        T

751   ATGCAGCGGTTGTCGCAGCCGGCGCACACTCGAAATCACTTGCTAATTCG   800
             T     T     T  T        T

801   CTAGGCGATGACATCCCGCTCGATACCGAACGTGGATATCATATCGTCAT   850

851   CGCGAATCCGGAAGCCGCTCCACGCATTCCGACGACCGATGCGTCAGGAA   900

901   AATTCATCGCGACACCTATGGAAATGGGGCTTCGCGTGGCGGGTACGGTT   950
                                  T     T  T  T     T

951   GAGTTCGCTGGGCTCACAGCCGCTCCTAACTGGAAACGTGCGCATGTGCT   1000
           T     T        T

1001  CTATACGCACGCTCGAAAACTTCTTCCAGCCCTCGCGCCTGCGAGTTCTG   1050

1051  AAGAACGATATTCCAAATGGATGGGGTTCCGGCCGAGCATCCCGGATTCG   1100
                                 T  T  T  T     T  T     T

1101  CTCCCCGTGATTGGCCGGGCAACCCGGACACCCGACGTAATCTATGCTTT   1150
        T  A        T  T     T  T

1151  CGGCCACGGTCATCTCGGCATGACAGGGGCGCCGATGACCGCAACGCTCG   1200
      T  T              T        T  T  A     T     T

1201  TCTCAGAGCTCCTCGCAGGCGAAAAGACCTCAATCGACATTTCGCCCTTC   1250

1251  GCACCAAACCGCTTTGGTATTGGCAAATCCAAGCAAACGGGTCCGGCAAG   1300

1301  TTAAGTGGGAATTCAAGCTTG   1321
      STOP
```

FIG.3B

```
  1  AGATCTCCATGGCTGAGAACCACAAAAAAGTAGGCATCGCTGGAGCCGGA  50
                                  G  G  T  T           T

 51  ATCGTCGGCGTATGCACGGCGCTGATGCTTCAGCGCCGCGGATTCAAAGT  100
          T  T  T     T  TT          A  T  T        G

101  CACCTTGATTGACCCGAACCCTCCTGGCGAAGGTGCATCGTTTGGGAATG  150
     T           T  A     A  A  T        T  T  C  T  C

151  CCGGATGCTTCAACGGCTCATCCGTCGTCCCTATGTCCATGCCGGGAAAC  200
      T  T           T  C     T  T  A           A

201  TTGACGAGCGTGCCGAAGTGGCTCCTTGACCCGATGGGGCCGTTGTCAAT  250
          T     T  A        T        A     T  A     C

251  CCGGTTCAGCTATTTTCCAACCATCATGCCCTGGTTGATTCGCTTTCTGT  300
        T        C                 T           T  CT  C

301  TAGCCGGAAGACCAAACAAGGTGAAGGAGCAGGCGAAAGCACTCCGCAAT  350
      T  T                          A  T  G        T  C

351  CTCATCAAGTCCACGGTGCCTCTGATCAAGTCATTGGCGGAGGAGGCTGA  400
                   T      T          C     T

401  TGCGAGCCATCTGATCCGCCATGAAGGTCATCTGACCGTATATCGTGGAG  450
        T     C  T     T  C        C  T     G  C

451  AAGCAGACTTCGCCAAGGACCGCGGAGGTTGGGAACTGCGGCGTCTCAAC  500
                           T              T  T

501  GGTGTTCGCACGCAGATCCTCAGCGCCGATGCGTTGCGGGATTTCGATCC  550
             T  T  A           T     A     T

551  GAACTTGTCGCATGCGTTTACCAAGGGCATTCTTATAGAAGAGAACGGTC  600
     T        T  C  C           A  C     C
```

FIG.4A

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```
  601   ACACGATTAATCCGCAAGGGCTCGTGACCCTCTTGTTTCGGCGTTTTATC   650
            C  C  C  A     T        T           T     C

  651   GCGAACGGTGGCGAATTTGTATCTGCGCGTGTCATCGGCTTTGAGACTGA   700
          T        A  G  C  G     T     T     A  C

  701   AGGTAGGGCGCTTAAAGGCATTACAACCACGAACGGCGTTCTGGCCGTTG   750
            C T  T  C  G  T  C  C     C     T     T  T

  751   ATGCAGCGGTTGTCGCAGCCGGCGCACACTCGAAATCACTTGCTAATTCG   800
               T     T     T  T        C  G  T        C  C

  801   CTAGGCGATGACATCCCGCTCGATACCGAACGTGGATATCATATCGTCAT   850
          T  T           AT G                 C  C     G

  851   CGCGAATCCGGAAGCCGCTCCACGCATTCCGACGACCGATGCGTCAGGAA   900
           C  C  A     T        T     A  T        T  T

  901   AATTCATCGCGACACCTATGGAAATGGGGCTTCGCGTGGCGGGTACGGTT   950
         G        T  T        G     T     T  T  T  A  C

  951   GAGTTCGCTGGGCTCACAGCCGCTCCTAACTGGAAACGTGCGCATGTGCT   1000
                   T     T  T              G     T  C  T

 1001   CTATACGCACGCTCGAAAACTTCTTCCAGCCCTCGCGCCTGCGAGTTCTG   1050
           C  T        T  GT G        T     T     C

 1051   AAGAACGATATTCCAAATGGATGGGGTTCCGGCCGAGCATCCCGGATTCG   1100
               T  C     G        T     T  A        A     C

 1101   CTCCCCGTGATTGGCCGGGCAACCCGGACACCCGACGTAATCTATGCTTT   1150
          T  A         T  T  T    T  T  A     T     C

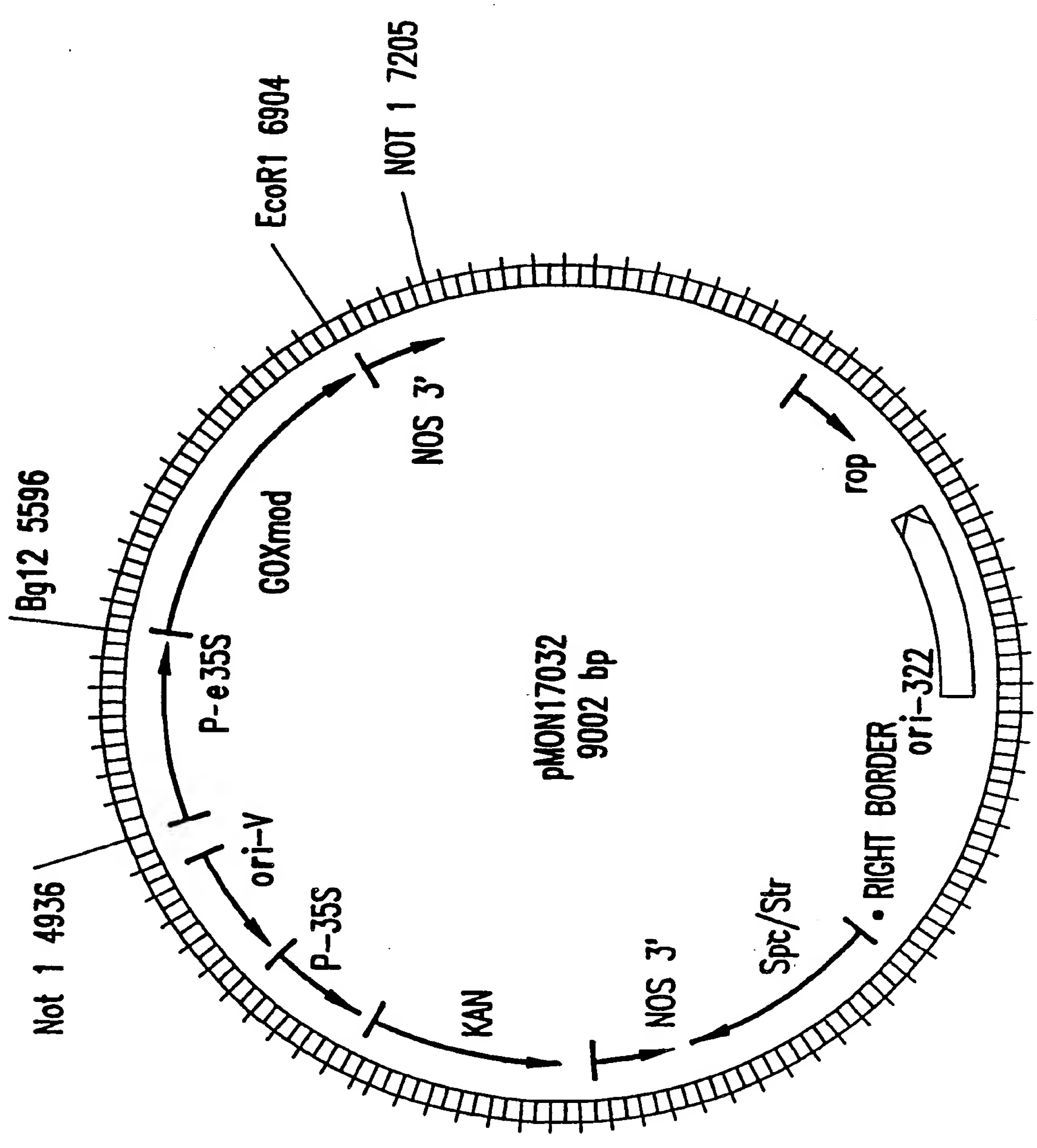
 1151   CGGCCACGGTCATCTCGGCATGACAGGGGCGCCGATGACCGCAACGCTCG   1200
           T        C     T     T  T  T  A           C

 1201   TCTCAGAGCTCCTCGCAGGCGAAAAGACCTCAATCGACATTTCGCCCTTC   1250
         T  T              T  G        T        C  T  A

 1251   GCACCAAACCGCTTTGGTATTGGCAAATCCAAGCAAACGGGTCCGGCAAG   1300
                  T   C        T  G           T     T   TC

 1301   TTAAGTGGGAATTCAAGCTTG   1321
        C
```

FIG.4B



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EcoR1 6904
NOT 1 7205
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GOXmod
NOS 3'
rop
ori-322
RIGHT BORDER
Spc/Str
NOS 3'
KAN
P-35S
ori-V

FIG.5

```
          B
          g
          l
          I
          I
          AGATCTCCACAATGGCTTCCTCTATGCTCTCTTCCGCTACTATGGTTGCCTCTCCGGCTC
     1    ---------+---------+---------+---------+---------+---------+ 60
          TCTAGAGGTGTTACCGAAGGAGATACGAGAGAAGGCGATGATACCAACGGAGAGGCCGAG

c'                  MetAlaSerSerMetLeuSerSerAlaThrMetValAlaSerProAlaGln -

          AGGCCACTATGGTCGCTCCTTTCAACGGACTTAAGTCCTCCGCTGCCTTCCCAGCCACCC
    61    ---------+---------+---------+---------+---------+---------+ 120
          TCCGGTGATACCAGCGAGGAAAGTTGCCTGAATTCAGGAGGCGACGGAAGGGTCGGTGGG

c'         AlaThrMetValAlaProPheAsnGlyLeuLysSerSerAlaAlaPheProAlaThrArg -

          GCAAGGCTAACAACGACATTACTTCCATCACAAGCAACGGCGGAAGAGTTAACTGCATGC
   121    ---------+---------+---------+---------+---------+---------+ 180
          CGTTCCGATTGTTGCTGTAATGAAGGTAGTGTTCGTTGCCGCCTTCTCAATTGACGTACG

c'         LysAlaAsnAsnAspIleThrSerIleThrSerAsnGlyGlyArgValAsnCysMetGln -

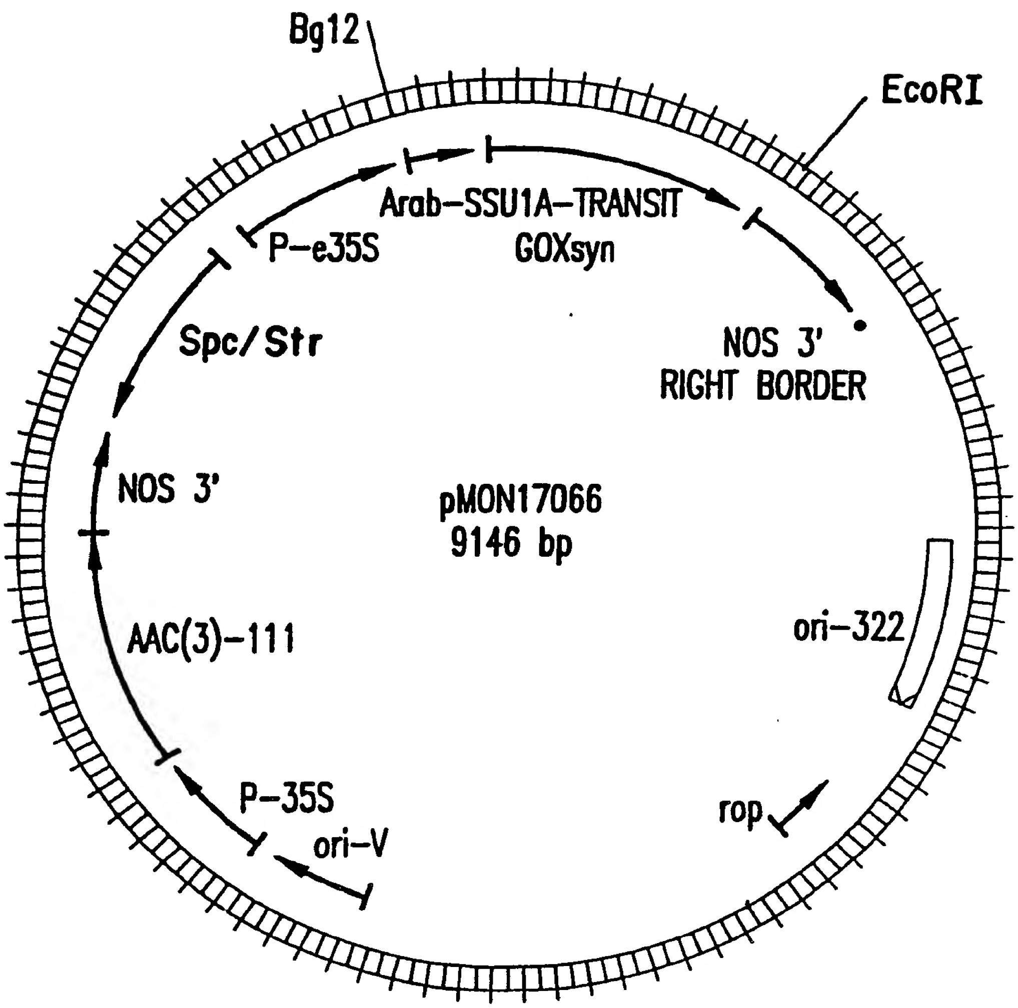
          AGGTGTGGCCTCCGATTGGAAAGAAGAAGTTTGAGACTCTCTCTTACCTTCCTGACCTTA
   181    ---------+---------+---------+---------+---------+---------+ 240
          TCCACACCGGAGGCTAACCTTTCTTCTTCAAACTCTGAGAGAGAATGGAAGGACTGGAAT

c'         ValTrpProProIleGlyLysLysLysPheGluThrLeuSerTyrLeuProAspLeuThr -

                                            N
                                            c
                                            o
                                            I
          CCGATTCCGGTGGTCGCGTCAACTGCATGCAGGCCATGG
   241    ---------+---------+---------+--------- 279
          GGCTAAGGCCACCAGCGCAGTTGACGTACGTCCGGTACC

c'         AspSerGlyGlyArgValAsnCysMetGlnAlaMet
```

FIG.6



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Spc/Str
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ori-V
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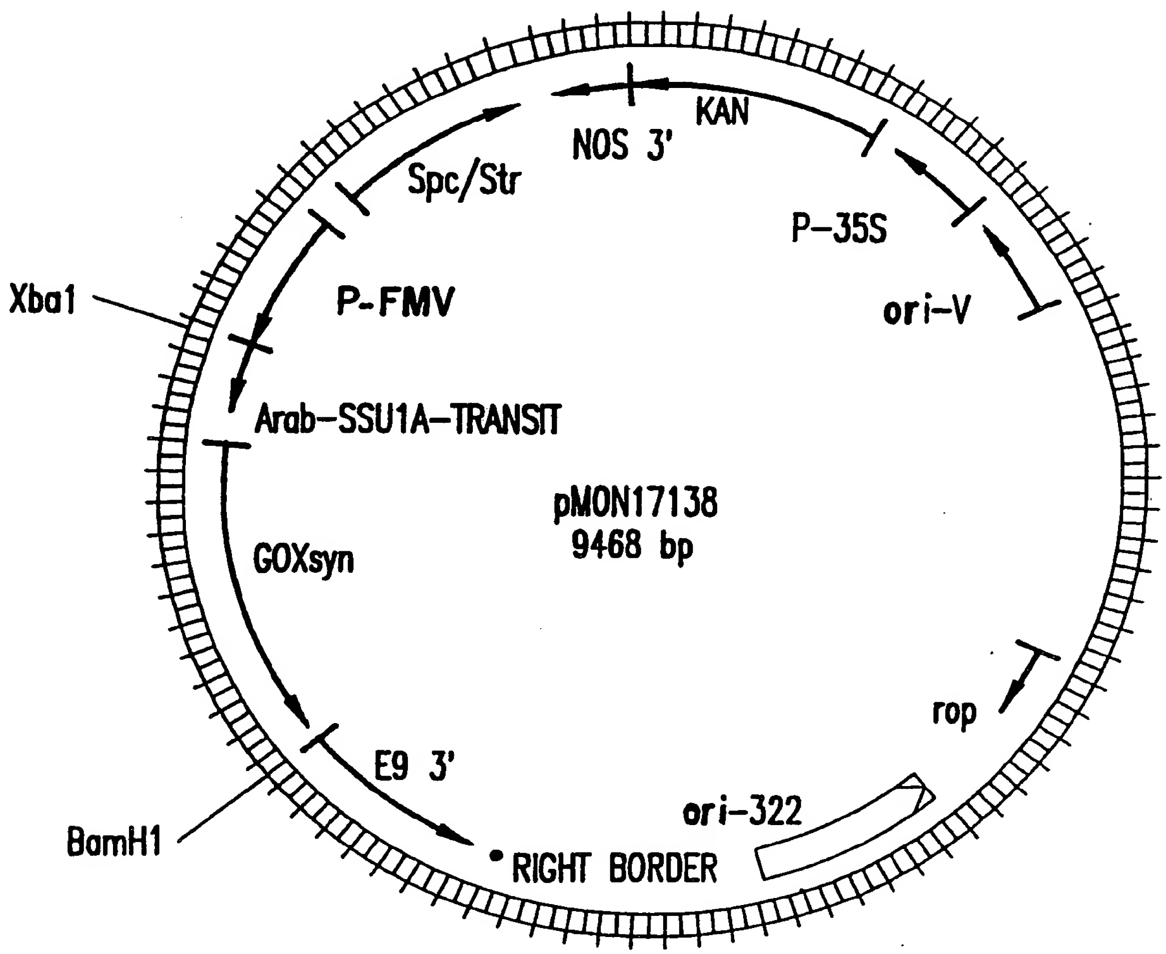
FIG.7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GOXsyn
rop
E9 3'
ori-322
BamH1
RIGHT BORDER

FIG.8

```
         B
         g
         l
         I
         I
         AGATCTATCGATAAGCTTGATGTAATTGGAGGAAGATCAAAATTTTCAATCCCCATTCTT
      1  ---------+---------+---------+---------+---------+---------+  60
         TCTAGATAGCTATTCGAACTACATTAACCTCCTTCTAGTTTTAAAAGTTAGGGGTAAGAA

         CGATTGCTTCAATTGAAGTTTCTCCGATGGCGCAAGTTAGCAGAATCTGCAATGGTGTGC
     61  ---------+---------+---------+---------+---------+---------+  120
         GCTAACGAAGTTAACTTCAAAGAGGCTACCGCGTTCAATCGTCTTAGACGTTACCACACG

                                      MetAlaGlnValSerArgIleCysAsnGlyValGln -

         AGAACCCATCTCTTATCTCCAATCTCTCGAAATCCAGTCAACGCAAATCTCCCTTATCGG
    121  ---------+---------+---------+---------+---------+---------+  180
         TCTTGGGTAGAGAATAGAGGTTAGAGAGCTTTAGGTCAGTTGCGTTTAGAGGGAATAGCC

c         AsnProSerLeuIleSerAsnLeuSerLysSerSerGlnArgLysSerProLeuSerVal -

         TTTCTCTGAAGACGCAGCAGCATCCACGAGCTTATCCGATTTCGTCGTCGTGGGGATTGA
    181  ---------+---------+---------+---------+---------+---------+  240
         AAAGAGACTTCTGCGTCGTCGTAGGTGCTCGAATAGGCTAAAGCAGCAGCACCCCTAACT

c         SerLeuLysThrGlnGlnHisProArgAlaTyrProIleSerSerSerTrpGlyLeuLys -

         AGAAGAGTGGGATGACGTTAATTGGCTCTGAGCTTCGTCCTCTTAAGGTCATGTCTTCTG
    241  ---------+---------+---------+---------+---------+---------+
         TCTTCTCACCCTACTGCAATTAACCGAGACTCGAAGCAGGAGAATTCCAGTACAGAAGAC
c         LysSerGlyMetThrLeuIleGlySerGluLeuArgProLeuLysValMetSerSerVal -

                          S
                          p
                          h
                          I
         TTTCCACGGCGTGCATGC
    301  ---------+--------
         AAAGGTGCCGCACGTACG

c         SerThrAlaCysMet
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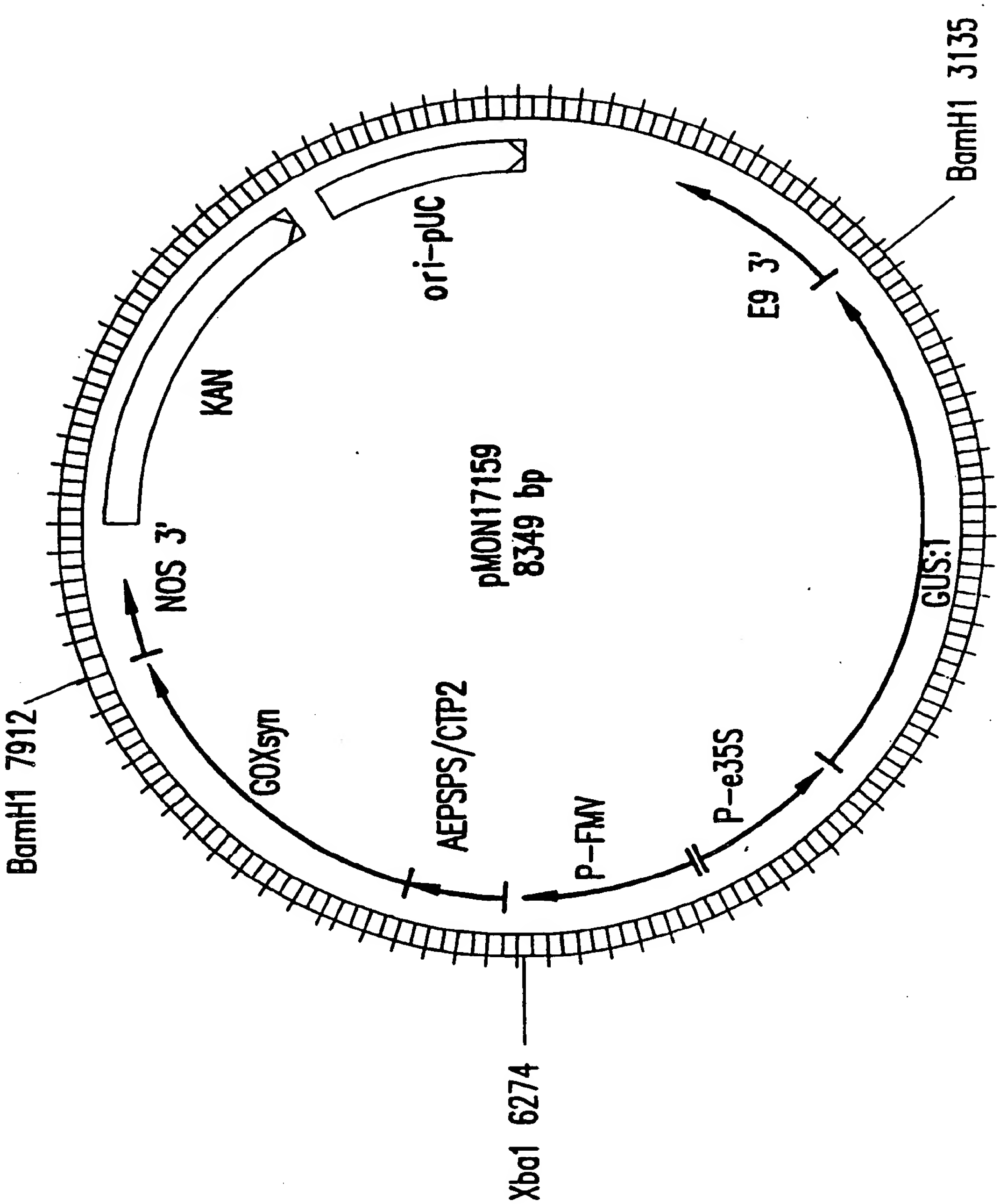
FIG.9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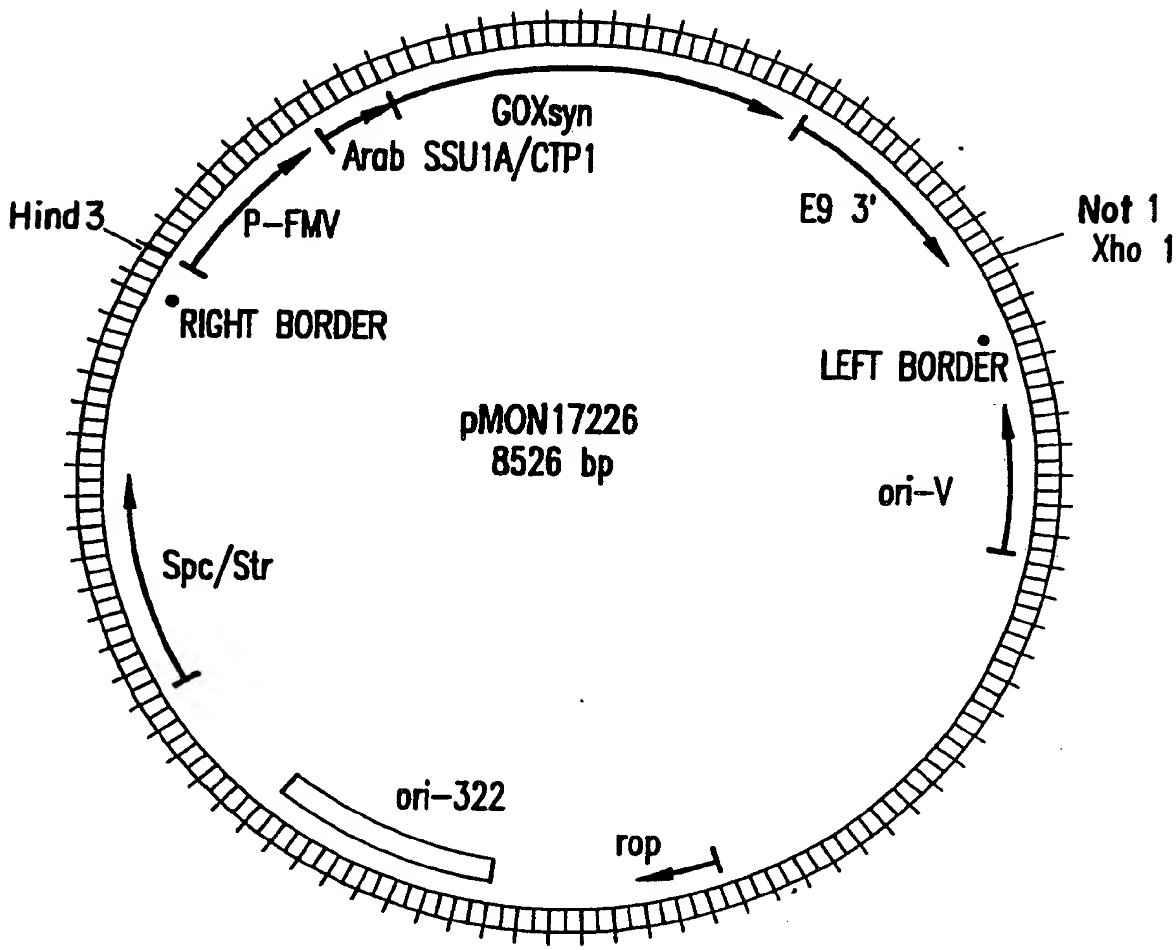
P-e35S
Xba1 6274

FIG.10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E9 3'
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Xho 1
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ori-V
Spc/Str
ori-322
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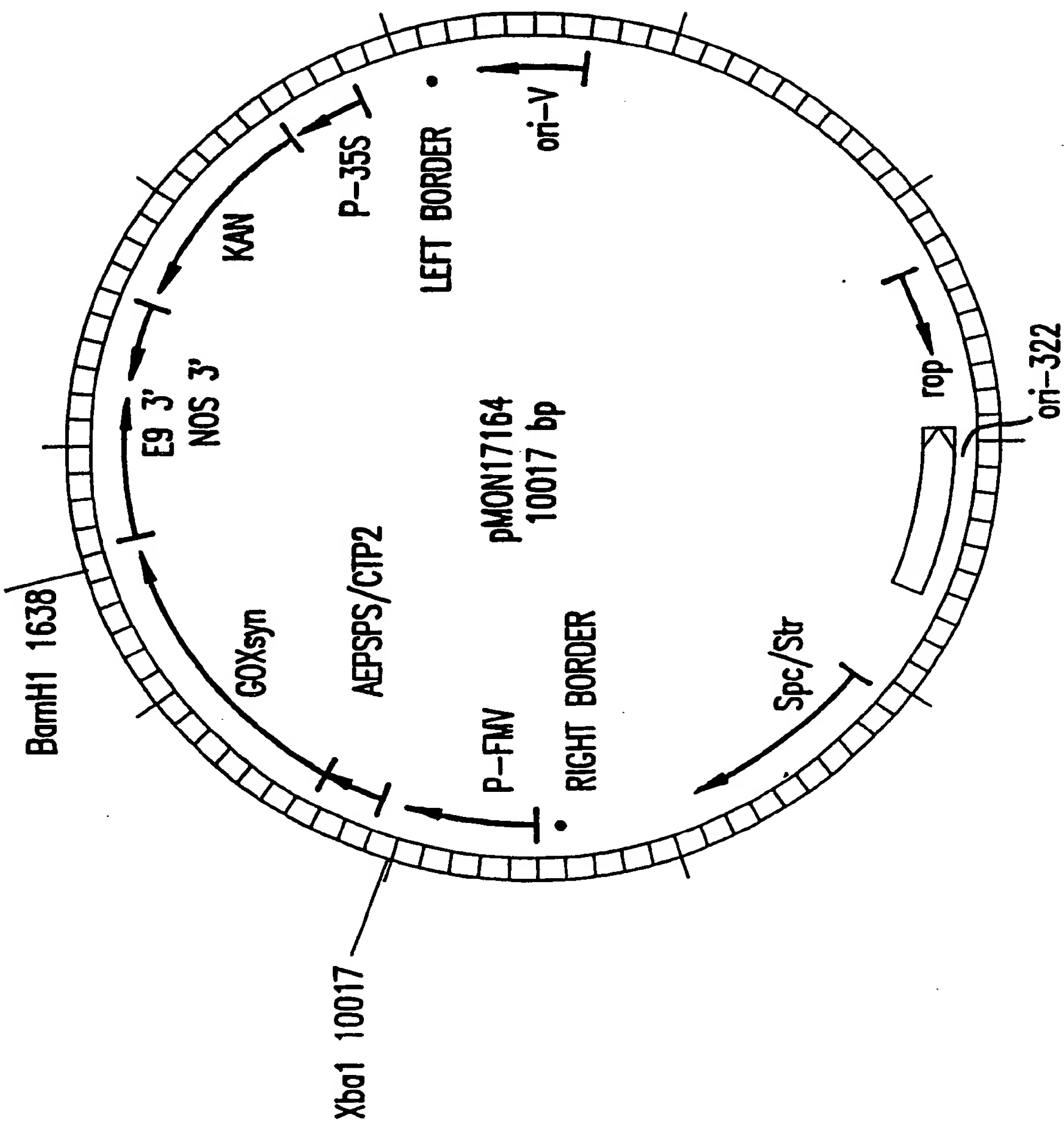
FIG.11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Xba1 10017
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P-FMV
RIGHT BORDER
E9 3'
NOS 3'
KAN
P-35S
LEFT BORDER
ori-V
pMON17164
10017 bp
rop
ori-322
Spc/Str

FIG.12

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/04514

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/53; C12N15/82; C12N9/06; C12N9/02
C12N5/10; A01H5/00; A01H1/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	APPLIED ENVIRONMENTAL MICROBIOLOGY vol. 54, no. 12, December 1988, pages 2953 - 2958; JACOB, G. S., ET. AL.: 'Metabolism of glyphosate in Pseudomonas sp. strain LBr' cited in the application see the whole document ---	1,21,22, 32
A	J. CELLULAR BIOCHEMISTRY vol. 13D, 1989, MEETING APRIL 1-7 1989 page 338; MCLEAN, P. A., ET.AL.: 'Toward herbicide resistant plants: cloning of the genes for glyphosate degradation from a soil organism, and their expression in E.coli' see the abstract M528 ---	2-20,31

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 NOVEMBER 1991	04. 12. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

2

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	TREND IN GENETICS vol. 4, no. 8, August 1988, pages 219 - 222; BOTTERMAN J.,ET.AL: 'Engineering herbicide resistance in plants' see page 221 left hand column last 2 paragraphs ---	2-20,31
A	APPLIED ENVIRONMENTAL MICROBIOLOGY vol. 54, no. 5, May 1988, pages 1293 - 1296; PIPKE R.,ET.AL.: 'Degradation of the phosphonate herbicide glyphosate by Arthrobacter atrocyaneus ATCC 13752' see the whole document ---	1,21,22, 32
A	TIBTECH vol. 8, no. 3, March 1990, pages 61 - 65; OXTOBY, E.,ET.AL: 'Engineering herbicide tolerance into crops' see page 64, column 3 - page 65, column 1 ---	2-20,31

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